MICROBIOLOGICAL CHARACTERIZATION OF 'HURMA' OLIVES GROWN IN KARABURUN PENINSULA

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Gözde Seval KARSLI

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We approve the thesis of **Gözde Seval KARSLI**

Examining Committee Members:

Assist. Prof. Dr. Ayşe Handan BAYSAL
Department of Food Engineering,
İzmir Institute of Technology

Assoc. Prof. Dr. Fatma Banu ÖZEN
Department of Food Engineering,
İzmir Institute of Technology

Assist. Prof. Dr. Nükhet N. DEMİREL ZORBA
Department of Food Engineering,
Çanakkale Onsekiz Mart University

12 July 2013

Assist. Prof. Dr. Ayşe Handan BAYSAL
Supervisor, Department of Food Engineering
İzmir Institute of Technology

Prof. Dr. Ahmet YEMENİCİOĞLU
Head of the Department of
Food Engineering

Prof. Dr. R. Tuğrul SENGER
Dean of the Graduate School of
Engineering and Sciences

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ABSTRACT

MICROBIOLOGICAL CHARACTERIZATION OF ‘HURMA’ OLIVES GROWN IN KARABURUN PENINSULA

Erkence variety olive (*Olea europea* L.) cultivar growing in the Aegean Region of Turkey is a naturally black olive. Debittered Erkence variety called ‘Hurma’ olive is an unusual olive type which is characterized by the sweet taste of its fruit. This olive which is grown mainly in Karaburun Peninsula differs from other varieties since it ripens on the tree losing its bitterness caused by phenolic compounds especially oleuropein. Thus, Hurma olives can be directly consumed from the tree without a further debittering process to make them edible.

Total aerobic mesophilic microorganisms, lactic acid bacteria (LAB), Enterobacteriacaae, Pseudomonadaceae, Staphylococcaceae-Micrococcaceae, moulds and yeasts were enumerated in the olive drupes, leaves and orchards’ air of Erkence and Gemlik cultivars during the maturation period. Moreover, bacterial microflora of Hurma olive fruit, leaf and orchards’ air were characterized in terms of DNA sequencing.

Microbial loads of naturally debittered ‘Hurma’ olive were higher when compared with Gemlik olive’s and non-debittered Erkence variety olive’s. But no Pseudomonadaceae, Staphylococcaceae-Micrococcaceae and LAB were detected in all samples.

Bacterial microflora genera of Hurma olive comprised of *Bacillus, Pantoea, Acinetobacter and Pseudomonas* while the other samples have also similar bacterial genera. The common genus found in all samples was *Bacillus*. Besides, more diversified genera were obtained from phylloplane and air microflora of Erkence variety olive orchard was substantially similar to bacterial phylloplane of leaf.

This is the first study about microbiological characterization of Hurma olive type and will lead up to new studies about it.

ÖZET

KARABURUN YARIMADASI'NDA YETİŞEN 'HURMA' ZEYTİNİNİN MİKROBİYOLOJİK KARAKTERİZASYONU

Türkiye' nin batısında yetişen Erkence çeşidi zeytin bitkisi (*Olea europea* L.) doğal siyah zeytindir. Alışılmadık tatlı tadı ile ile karakterize edilen acılığını kaybetmiş Erkence çeşidi 'Hurma' zeytin genellikle Karaburun yarımadasında yetişir. Diğer çeşit zeytinlerden fenolik bileşiklerin özellikle oleuropeinin sebep olduğu acılığı, dalında olgunlaşması sırasında kaybetmesi ile ayrılır. Böylece hasat edildiğinde herhangi bir acılık giderme işlemine gerek duymadan doğrudan tüketilebilir.

Erkence ve Gemlik çeşitlerine ait zeytin, yaprak ve bahçe havası örneklerinde olgunlaşma süresinde, toplam aerobik mezofilik mikroorganizma, laktik asit bakterileri (LAB), Enterobacteriacaae, Pseudomonadaceae, Staphylococcaceae-Micrococcaceae, küf ve maya sayımları yapılmıştır. Ayrıca, Erkence çeşidine ait örneklerin DNA dizi analizi ile bakteri mikroflorası karakterize edilmiştir.

Doğal olarak acılığını kaybeden Erkence çeşidi 'Hurma' zeytinle, Gemlik ve acılığını kaybetmemiş Erkence cinsi zeytin, bu mikroorganizma gruplarının yükü açısından karşılaştırıldığında, Hurma zeytindeki mikrobiyel yükün, Gemlik çeşidi ve acılığını kaybetmemiş Erkence çeşidi örneklerine göre daha fazla olduğu bulunmuştur. Fakat örneklerin tamamında Pseudomonadaceae, Staphylococcaceae-Micrococcaceae ve LAB saptanamamıştır.

Hurma zeytindeki bakteriyel mikroflora *Bacillus, Pantoea., Acinetobacter* ve *Pseudomonas* cinslerinden oluşmakta iken, diğer örnekler de benzer bakteri cinsleri gözlenmiştir. Tüm örnek materyallerinde ortak bulunan cins *Bacillus'* tur. Ayrıca, yaprak yüzeyindeki habitatta daha çeşitli cinsler elde edilmiş ve bahçe hava mikroflorasının yaprak yüzeyindeki bakteriyel mikroflora ile büyük ölçüde benzer olduğu gözlemlenmiştir.

Bu çalışma, doğal olarak acılığı kaybetmiş Erkence çeşidi zeytinin mikrobiyolojik karakterizasyonu olması açısından ilk olacak ve hurma zeytinle ilgili diğer çalışmaların önünü açacaktır.

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CHAPTER 1

INTRODUCTION

Table olive production is mainly associated with the Mediterranean countries (Pereira et al., 2008). The International Olive Oil Council reported the major producer in order of decreasing production rates as Spain, Egypt, Turkey, Syria, Greece, Morocco, Italy and Portugal according to the 2009/10 seasons' numbers. In the relevant season, Spain (22%), Egypt (18.5%) and Turkey (17.6%) were the leading producers through the worldwide with the 1,291.6 tonnes of 2,209.5 tonnes of table olive production. The last six seasons' average production of table olive of Turkey reached to 318,300 tonnes (IOOC, 2012). According to 2009/10 season numbers, 66.6 % of table olives were consumed in Turkey while the rest was exported (IOOC, 2012). In 2010, olive orchards covered 791.000 ha of land in Turkey; 72% of total crop area planted to produce olive oil, versus 28% for table olive production. With the expanding acreage of olive by 129,000 ha over the last five crop years, it is expected that olive orchard will reach an area of 1,000,000 ha by 2015 (IOOC, 2012).

Olive production provides economic input for producer from Aegean, Marmara, Mediterranean, South-eastern Anatolia and Black Sea regions where olive is grown and different olive types grown throughout Turkey have its own distinguishing characteristics (IOOC, 2012). Aegean region is the leading producers among the all producer regions, and 62.8 % of table olive production was met by Aegean region in 2007. If it is evaluated on a city-by-city basis, İzmir came after Aydın in second place with the 13.6% of table olives (Öztürk et al., 2009). Olive cultivars grown in Aegean region are Ayvalık (Edremit), Çakır, Çekişte, Çilli, Domat, Edincik, Erkence, Gemlik, İzmir sofralık, Kiraz, Memecik, Memeli and Uslu (OOPC,2013). Naturally debittered Erkence olive varieties –Hurma olives-, the subject of this thesis is grown in the restricted area of northwest part of İzmir where Karaburun, Urla and Çeşme are. It has specific characteristic of lose its bitterness which is caused by oleuropein and it turns black to dark brown color during maturation period on the tree. Thus, it can be directly consumed when it is harvested that is why it is called as 'Hurma' olive. Degradation of bitterness leading component –oleuropein- was associated with the hydrolysing activity

of a fungus which is *Phoma olea* (Buzcu, 1969). Since its bitterness component lessens during maturation on the tree, it does not required further processing such as debittering and brining in order to store it. It is also notified that a similar self-debittered olive is grown in Tunisia which is Dhokar olive variety. Dhokar olive fruits are famous for by the lack of the bitter taste before reaching maturation (Jemai et al., 2009). Similar olive variety –Throuba- is grown mainly on the island of Thassos in northern Greece. Throuba olives completely maturates when their superficial color turns to brown while still on the tree by means of *Phoma oleae,* a natural safe fungus. As a result, they have a bittersweet taste and can be consumed straight from the tree (Panagou, 2006). Naturally debittered 'Hurma' olive has gained approval by consumers due to its organoleptic characteristics and it is a very significant product economically for its growers.

Olives are one of the indispensable components of the Mediterranean diet (Pereira et al., 2008) which has a reputation to prevent the suffering from certain diseases such as cardiovascular diseases and some kind of cancers owing to a high level of monounsaturated fatty acids (Boscou, 2012). Increasing interest to the Mediterranean diet has lead to consumption of natural food products such as olive and olive oil. Unprocessed, salt-free 'Hurma' olive satisfies this demand since it is a preferable alternative to processed table olives.

In the literature, studies of microbial characterization of olive as a raw material is very limited and existential studies are concentrated on the characterizations of olive oil (Gürdeniz et al., 2007 and 2008) and processed table olives (Hurtado et al., 2008, Pereira et al., 2008).

In this study it was aimed to the bacterial population of naturally debittered Erkence olives and to determine if there is a relationship between the environment where olives are grown and this was achieved by characterization of microbial population of naturally debittered Erkence olives, olive leaf and orchard's air samples during its maturation period. Besides during maturation, microbial population of naturally debittered Erkence olive was compared to Gemlik olives. This study is also a novel research to characterize microbial flora of the naturally debittered Erkence olive. In this respect this study is the first in the literature.

CHAPTER 2

LITERATURE REWIEV

2.1. The Olive Plant and Its Brief History

The olive has been always a part of the Mediterranean civilization since ancient times (Sibbert and Ferguson, 2004). Cultivation of the olive tree probably started about 5000 years ago in the Middle East, Mesopotamia and Syria, carried later to south and west through Palestine and Anatolia (Kailis and Harris, 2007). In later period, it was introduced to the other land of the Mediterranean basin by means of the Romans and then the Arabs. Todays, still the Mediterranean region is the first in production of olives. Even if it was taken by colonist or explorer to the England and the eastern United States, olive cultivation did not reach to success in that land because of unsuitable climatic conditions (Sibbert and Ferguson, 2004).

The Mediterranean climate is the best to grown of olive tree with a long, hot growing season and a relatively cool winter with minimum temperatures above the lethal limit (Yada and Harris, 2007). As a consequence of strict requirement of climatic conditions, the olive is widely distributed in both hemispheres between the latitude 15 to 35^{o} South and 25 to 45^{o} North in where olive can be produced successfully except Mediterranean basin where Mediterranean climate conditions are dominated such as South Africa, Australia and South America (Sibbert and Ferguson, 2004).

The olive belongs to the family of Oleaceae, genus *Olea* and to the species *Olea europaea,* L. which is the species of *Olea* is distributed in the tropical and sub-tropical regions of the world. The edible table olives and olive oil are produced only from this species of *Olea* (Fernández, Diez and Adams, 1997).

The olive is drupes that comprise of three parts are the epicarp or skin, the mesocarp or flesh/pulp and woody endocarp or pit (Fernández, Diez and Adams 1997). It has the characteristics of having a low sugar concentration between 2.0-6.0 %, a high fat content between 9-30% and the bitter taste cause from the glucoside of olive, oleuropein, which belongs to the *Oleaceae* family and can be found in other plants such

as *Gentianaceae* and *Cornaleae* (Omar, 2010 and Arroyo-López, et al., 2010). These characteristics are lead not to be suitable for consumption directly from the tree before being applied a series of processes to debitterring and are depend on the ripeness of olive and olive variety (Arroyo-López, et al., 2010).

2.2. World Consumption and Production of Table Olives

The most of the olive production in the world is gathered in countries around the Mediterranean Sea. The all producer Mediterranean countries are responsible from the 91% of production of table olives with the 2.086.900,0 tonnes in the world. Among these countries Spain, Eygpt and Turkey are the top three producers. Likewise the production, the most of consumption is occurred in the Mediterranean countries which consume 71% of table olives in the world. Eygpt, Turkey and Spain are the main consumers of table olives. The geographic distribution of world table olive production and consumption data are represent in the Table 1.1

Turkey is in the third place in production whilst in the consumption it is in the second place based on the data published on IOOC 2012 activity report. Increasing production of table olives in the recent crop seasons lead to increasing exportation of table olives also. Thereby, booming exportation in the last decade expands the Turkish table olives sector (IOOC, 2012).

2.3. Definitions of Table Olives

The Unified Qualitative Standard Applying to table olives define them as a product; "prepared from the sound fruits of varieties of the cultivated olive tree (*Olea europaea* L.) that are chosen for their production of olives whose volume, shape, flesh-to-stone ratio, fine flesh, taste, firmness and ease of detachment from the stone make them particularly suitable for processing; bitter taste of olive is removed and preserved by natural fermentation, or by heat treatment, with or without the addition of preservatives; packed with or without covering liquid" (IOOC, 2004).

Conformably, Turkish Food Codex defined the table olive as following: suitably debittered fruits of cultivated olive tree *(Olea europaea sativa*) that are produced with fermentation or no fermentation, if necessary lactic acid and/or the other additives are

added, pasteurized or sterilized or not applied both heat treatments (Turkish Food Codex, Table Olive Declaration, 2008/24).

According to the Turkish Food Codex, the following definitions are given for the types of table olives' ripeness degree:

Green olives are harvested before ripening; colors are change from green to greenish yellow with the normal size of olive fruits,

Turning color olives / rose olives are rose, wine-rose or light brown colored fruits, harvested before the full ripeness period,

Black olives are harvested just before full ripeness stage or when they are reached to full ripeness. Their colors are ranged from black, blackish purple, greenish black, dark brown to rose-black.

Removing the bitterness of olive completely or partially is the foremost purpose of processing to make it acceptable to consume (Fernández, Diez and Adams 1997).

Table 2.1 Distribution of world table olive consumption and production geographically (Source: International Olive Oil Council (IOOC) 2012)

	Production (%)*	Consumption (%)*
Spain	22.71	7.87
Eygpt	17.64	14.10
Turkey	13.94	10.82
Syria	6.29	5.30
Greece	4.81	1.05
Morocco	4.30	1.46
Italy	2.79	5.75
Tunisia	0.83	0.79
Others	8.57	28.49

* Average percentages between seasons 2006 and 2011.

2.4. Structure of Olive

The olive fruit is an oval shaped drupe, comprised of two major structural parts: the pericarp and the endocarp containing the seeds. The pericarp is separated into two parts that are the epicarp (skin) and the mesocarp (pulp) (Hashim et al., 2005). When

the whole olive drupe is the subject, it is composed of 50% of water, 22% of oil, 19.1% of carbohydrates, 1.6% of proteins, 5.8% of cellulose and 1.5% of minerals (Zamora et al., 2001).

As mentioned above, the pericarp is protective tissue against external damage, fungal growth and insects' infestation as an impermeable layer that accounts for 1.0-3.0% of the drupe weight. Thus, skin of olive is covered by a layer wax whose weight is around 45-70% of the skin. Colour of skin is green at beginning of development because of abundance of chlorophyll, throughout the maturation closes to end, changing to pale yellow, pink, purple and black. Range in color is depended on the concentrations of the chlorophyll, carotenoids and anthocyanins the major pigments of olive fruit (Therios, 2009).

The mesocarp comprises of the flesh of olive which is the edible part of table olives. It accounts for 70-80% of the whole drupe. The 70-75% of weight of the flesh is composed from water and the fat content changes between 14-15 % of green olive while it reaches to 30% in black olive. Generally, the table olives oil content is desired to be low, otherwise the high concentration of oil lead to some problems such as damage the consistency and preservation of the processed fruit. The free organic acids fraction found in olives is represented with oxalic, succinic, malic, and citric acids (1.2-2.1% of dry flesh), together with varying high amounts of free fatty acids(Bianchi, 2003). Besides the sugar content ranges between 3.5-6.0% of flesh and glucose, fructose, mannitol and saccharose are present in the flesh. As the maturation develops, the sugar concentration decreases. The smallest ratio in the flesh belongs to protein; 1.5-2.2% of the fruit is protein. Besides, polysaccharides and pectic substance are existed in the mesocarp tissues that are the important components of intercellular lamellae which are responsible for texture of the olive flesh and have a cementing function. These substances are the substrates for pectinolytic enzymes as a result of activity of those enzymes hardness of texture of the fruit lessens (Bianchi, 2003).

The endocarp in another name the stone, accounts for the 10-27% of the olive weight and the surrounded seed comprises 2-4% of the weight (Therios, 2009). The seed involves 22-27% oil while the other woody part of the stone contains %1 of oil. Some important characteristics of the endocarp can be listed with the size, weight, conformation of the stone and easy separation from the flesh that determines the quality of final product (Bianchi, 2003).

2.5. Phenolic and Lipid Content of Olive

When the table olive production is considered, phenolic compounds in olive is the major factor. Since the main consideration is that removal of phenolic compounds to make a desirable and palatable product. The olive fruit is rich in phenolic compounds that comprise 1-14% of weight of dry flesh. The phenolic compounds have beneficial effect on human health and the plant. They have role in defence mechanism of plant against deleterious pathogens and infesting organisms. Additionally it plays a role in browning reaction as a substrate, have nutritional, sensory characteristics as well (Bianchi, 2003). On the other hand, some studies show that phenolic compounds in olive and olive leaf attributed to human health in various ways;

- They have adverse effect on the promotion and progression of carcinogenic cells,
- They have anti-inflammatory effect by blocking lipoxygenase activity,
- They have antioxidant role in preventing lipid oxidation, lead to agumentation the ability of low density lipoprotein to resist oxidation,
- They have antimicrobial effect on Gram-negative and Gram-positive bacteria. It was studied that olive leaf extract has also antimicrobial activity by means of oleuropein. This effect emerges from the distribution of cell peptidoglycans and damaging of bacterial membrane,
- Antiviral effect of oleuropein in olive and olive leaf extract was studied against respiratory system virus and they inhibit activity of the viruses that affect the respiratory system. (Omar, 2010).

Phenols are secondary metabolite of plants having at least one hydroxylated aromatic ring. Oleuropein and ligstroside are major phenols found in olive and olive leaf tissue. Phenolic compounds in olive generally accumulate in the skin and around seed (Charoenprasert and Mitchell, 2012). Major phenolic compound in olive is oleuropein which is a water soluble phenol, with ligstroside, verbascoside and simple C_6-C_2 phenols, such as 4-hydroxytyrosol, tyrosol, as the glucosides or aglycones, and flavonoids are constituted the phenolic compounds of olive (Bianchi, 2003). The maturity degree, part of fruit, variety of olive and season are effective on the content of and concentration of phenolic composition. During the maturation period of olive, an

external intervention to olive tissue happens by pathogens, insects or mechanical damage, the enzyme *β*-glucosidase present in microorganisms hydrolyzes the convertion oleuropein to aglycone (Figure 2.1) (Charoenprasert and Mitchell, 2012).

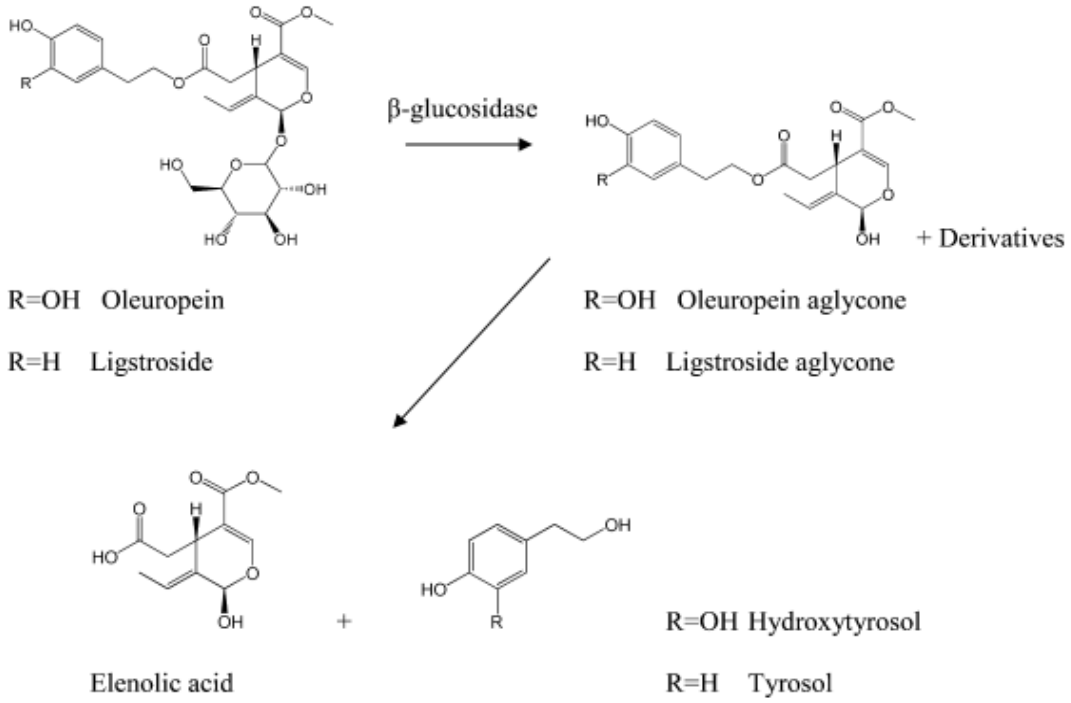

Figure 2.1 Hydrolization routes of oleuropein and ligstroside
(Source: Charoenprasert and Mitchell, 2012)

Phenolic contents can be classified into four major subgroup; phenolic acids: caffeic acid, coumaric acid, ferulic acid,vanillic acid etc., phenolic alcohols: hydroxytyrosol, tyrosol and their glucoside forms, flavonoids: luteolin-7-glucoside, cyanidin-3-glucoside, apigenin-7-glucoside, quercetin-3-rhamnoside and luteolin, secoiridoids: oleuropein, ligstroside that are found all olive varieties but, demthyloleuropein, verbascoside, ligustroside and cornoside are not found all varieties of olive so they can be used as varietal marker (Charoenprasert and Mitchell, 2012, Soler-Rivas et al,. 2000).

Throughout maturation on tree, olive passes three main phases that are:

1. Growth phase in which, oleuropein accumulates in the tissue of olive and reaches in content about %14 of dry weight of olive.

2. Green maturation phase: concentration levels of chlorophyll and oleuropein decline while derivatives of oleuropein glycoside concentration levels incline.

3. Black maturation phase in which ongoing reduction in oleuropein levels and appearance of anthocyanin occur (Yildiz and Uylaser, 2011).

Lipid composition of olive fruit is depending on maturity, variety and growth conditions (Kutlu and Şen, 2011). As the maturity develops lipid content increases (Nergiz and Engez, 2000). Deposition of lipid starts from end of the July and continues to October-November and reaches maximum level at the end of this period (Kutlu and Şen, 2011). Maturation resulted with concomitant decrease in oleic and palmitic acid while increment occurs in linoleic acid (Nergiz and Engez, 2000). When the olive is at the overripe stage, the ratio of oleic acid to linoleic acid decreases due to increase in linoleic acid amount (Conde et al, 2008). In addition free fatty acid level increases during maturation as a result of increment in enzymatic activity, especially in lipolytic enzymes (Salvador et al., 2001). In the study of Kutlu and Şen (2001), Gemlik olives grown in Alaşehir-Manisa were studied in whose composition mostly palmitic, stearic, oleic, and linoleic acids were found.

2.6. Production of Table Olives

Olives are significant agricultural products in the Mediterranean countries particularly it is economically important product. Raw olives cannot be eaten because of existence of bitter phenolic component, oleuropein. Processing of raw olives is required to diminish its bitterness. In this purpose, they are exposed to steep in water, brine or dilute alkali, or they are dried, salted or heated (Kailis and Harris, 2007).

Elaboration of olives are various to make them more palatable but fundamentally three methods to produce table olives are adopted in the international market which are Spanish style green olives, Greek style naturally black olives, and California style black ripe olives (Panagou et al., 2013).

Spanish style green olive production is the most applied method to produce table olives in which yellowish green to green olive drupes are harvested and graded in size and then are immersed into a dilute solution of sodium hydroxide (NaOH) (1.3-2.6% w/v food grade NaOH in water) during several hours. The concentration of alkali solution is depended on process temperature and the variety of olives. As temperature of process rises, penetration of alkali solution into the flesh gets easier. By means of alkali treatment, removal of oleuropein substantially is accomplished by penetration of NaOH

into the tissue of olive and it leads to augment of permeability of olive tissue in both way interchange of soluble substances. This debitterring step is followed by washing cycles to eliminate the excess of NaOH and olives are placed into sodium chloride solution where lactic acid fermentation comes about by lactic acid bacteria which produce desired organoleptic characteristics for this style of olives. Afterwards the fermentation is completed, olives are graded and sorted, if desired they are stuffed and finally packed (Arroyo Lopez et al., 2010, Kallis and Harris, 2007).

In the process of Californian type turning color olives, unreached to the complete ripeness are harvested that are mostly green color or at the turning stage of color to black whilst the oil formation of olive is not completed and pulp is still firm. In order to produce Californian type olives, dilute NaOH treatment, washing by water and aeration are followed each other for several times and this aerobic treatment cause to change in texture of flesh. During consecutive lye treatments, progressively olive skin and flesh become darker. This change in color is related to oxidation and polymerisation of phenolic content. When the desired color obtained, by way of solution of ferrous gluconate or lactate is added and color is fixed. Finally, product is canned in brine and autoclaved (Bianchi, 2003 and Arroyo-Lopez et. al., 2010).

When Greek style naturally black olives are considered, olives are harvested at the stage of completely ripeness or just before full ripeness. These olives are exposed to spontaneous fermentation during brining them into 8-10% of NaCI solution. The prevailing microflora of brine where the fermentation takes place, is comprised of Gram negative bacteria, lactic acid bacteria and yeasts (Panagou et al., 2011). This ecosystem of olive is affected by some important factors that are the pH, water activity of olive fruit, presence of nutrients and their distribution through the tissue, interaction of prevailing microflora, amount of phenolic compounds and organic acids of olive itself, temperature of fermentation and salt concentration of brine (Campaniello et al., 2005). Since there is no alkali treatment, the diffusion of fermentable compounds through the skin takes long time so the fermentation progress slowly. In the Greek style process, removing of phenolic compounds is carried out just by solubilisation of the oleuropein into the brine and equilibrium is reached after 8-12 months (Sanchez-Gomez et. al., 2006).

On the other hand, Thruba-style table olive production is important for similarity to Hurma olive. This olive variety is grown in a limited area like Hurma olive. They are

washed properly after harvesting and dried under sun to remove most of their moisture. With the addition of small quantity of salt, their organoleptic characteristics become more desirable. Generally the final product is packed in plastic bags or in tin cans with the olive oil, can be packed without addition of salt. Because of limited production, this product cannot meet the most of the demand of people who are suffered from cardiovascular or renal diseases (Fernández, Diez and Adams 1997).

2.7. Erkence and Gemlik Olive Varieties

Erkence and Gemlik olive varieties were used in this study as a materials. They are one of the most commonly cultivated olives in Turkey. They are important cultivars agronomicaly and economically.

Erkence: This variety is known as very vigorous with about 3,000,000 cultivated trees. Its productivity is not stable and medium. Oil productivity of this variety reaches up to 25 % so major aim to cultivate this olive variety is to produce olive oil. Additionally, green and black table olives can be produced from Erkence olive variety. Fruit is seperated from tree easily; therefore, generally before harvest fruits drop themselves.

Gemlik: Most of the black olives produced in Turkey are from this variety. It has a high and stable production capacity. With early ripening, fruits look glossy black color and have good taste and texture. Since it has high oil productivityof about 29 %, olive oil can also be produced and besides due to nice taste and texture table olives are also produced,. Therefore, Gemlik olives are mentioned as dual-purposed olives. Like Erkence, it is freestone and flesh to stone ratio is 5.6 (IOOC, 2012).

2.8. Hurma Olive

Naturally debittered Erkence olive variety which is called as ‘Hurma olives’ are naturally debittered black olives grown in a limited area where of the coast side of the Aegean region especially, northwest part of İzmir, Karaburun, Çeşme and Urla. They start to maturate late of October until late of December or January when they are fully ripe. Since this variety is easy to detach from tree they drop on the ground. Olives are

generally collected by hand from ground. While Hurma olive maturates on the tree it losses its bitterness caused by phenolic compounds especially oleuropein. Hurma olive has specific characteristic and turns dark brown to black color (Figure 2.2) during maturation period on the tree. Thus, it can be directly consumed when it is harvested that is why it is called as 'Hurma' (date in Turkish) olive. Hence, it does not require to undergo debittering process to make it edible by removing its bitter components. The area where Hurma olives are grown and also climatic conditions affect the formation of Hurma olive. Unique characteristic of Hurma olive makes it an important product economically and agriculturally.

Figure 2.2 Images of Hurma Olives

2.9. Microflora of Olive Fruit

Studies of raw olive fruits' microbiological characterization are scarce in the literature. But there are many studies about microbiological characterization of fermented and processed olives (Panagou, 2006, Nychas et al., 2002, Campaniello et al., 2005, Asehraou et al., 1992).

In raw black olives, a various and plenteous epiphytic microflora accommodate and this microflora contain many potential spoilage microorganisms and an extremely low number of lactic acid bacteria (Borcakli et al., 1993).

In a study of Fakas et al. (2010), Greek 'Amfissis' olive fruits were studied. Different batches of olives belonging to harvest times of the beginning (batch A) and the end (batch B) were analyzed. For two batches, molds: *Fusarium* and *Penicillium spp.*, yeasts from the genera *Candida, Cryptococcus, Pichia* and *Rhodotorula* were detected. As Gram-positive bacilli and cocci *Actinomyces* spp. (batch A),

Corynebacterium sp. (both batches) were mainly identified. On the other hand, Gram negative bacilli *Pseudomonas*, *Vibrio spp*. were identified in batch B and only the genera *Pseudomonas*, *Acinetobacter*, *Achromobacter* were identified in batch B.

On the other hand, for production of hot air dried olives of Thassos variety, olives at the stage of over ripeness were microbiologically analyzed before they were processed (Mantzouridou and Tsimidoou, 2011). In the raw olive samples of Thassos black olives, total viable counts, lactic acid bacteria and yeasts were enumerated (log 6.6 cfu/g, log 3.3cfu/g, log 4.7 cfu/g, respectively) but *Enterobacteriaceae, Staphylococcus, Bacillus* and *Clostridium* remained below log 1 cfu/g.

2.10. Microflora of Olive Leaf

Bacteria, filamentous fungi and yeast composed of the microorganisms inhabiting directly on the plant surface; called as phylloplane (Lee and Hyde, 2002), while the habitat adjacent to the plant surface; called as phyllosphere (Mohapatra, 2008). Population and range of phylloplane microorganisms are dependant on various factors that are temperature, relative humidity, exposure time of leaf with water droplets, speed of wind, light intensity, presence of pesticides residuals, air pollution, age of leaf, season, external nutrient and interaction between different microorganisms (Bakker et al., 2002). For example, varieties of microorganisms grown on young leaves are much more than those from old leaves (Ercolani, 1991, Thompson et al., 1993). Besides, the bacterial diversity is affected by seasonal change, throughout in the warmest and driest months the diversity of phylloplane is at the lowest level while it is at the highest level in the coolest and rainy months (Lindow and Brandl, 2003).

The unique study of olive leaves' microorganisms of phylloplane belongs to Ercolani (1991). In this study, during six growing seasons, olive leaves were investigated microbiologically. Culturable aerobic bacteria were isolated and identified in respect of the determination of the bacterial community structures on leaves of the same age at a given time intervals in growing seasons. Overall six year identification results showed that *Pseudomonas syringae* comprised of the 51% of the total population followed by *Xanthomonas campestris* 6.7% *and Erwinia herbicola, Acetobacter aceti, Gluconobacter oxydans, Pseudomonas fluorescens, Bacillus megaterium, Leuconostoc*

mesentroides subsp. dextranium, Lactobacillus plantarum, Curtobacterium plantarum, Micrococcus luteus were present in the descending order of occurrence.

2.11. Olive Orchard's Air Microflora

Hitherto conducted studies about air microflora are not relevant to olive orchards. Microorganisms are transferred to the tree and fruit via air, insects and birds. Very low amount of nutrient availability in the air hampers the proliferation of microorganisms. Moreover affecting factors of the diversity of microflora of air are weather parameters such as temperature, humidity, wind and duration of insolation (Tuszynski and Satora, 2003).

The predominant microorganisms observed in the atmosphere are moulds which mostly belong to *Cladosporium, Alternaria, Penicillium, Aspergillus* and *Fusarium* genera up to 20000 cfu/m^3 and yeasts up to 60000 cfu/m^3 with majority of *Candida* which without fermentation ability. Moreover bacteria are found in the air flora up to 10000 cfu/ m^3 and the prominent microorganisms are spore forming *Bacillus* and aerobic micrococci (Tuszynski and Satora, 2003).

Tuszynski and Satora (2003) carried out a study about qualitative and quantitative composition of soil, air, fruit and tree microflora of plum orchard located in submontane region of Lacko in Poland. It was concluded that the microflora prevailing of plum trees, orchard's soil and air microflora were related to each other. The representative members of genera present as dominant groups were *Bacillus, Rhodotorula, Candida, Trichoderma* and *Penicillium.*

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1 Sample Collection

During maturation periods of Erkence and Gemlik cultivars, olives and olive leaves were obtained from two different orchards. As 'Hurma' olive, Erkence olive cultivar and their leaf samples were collected from Eğlenhoca village in Karaburun which is situated at latitude 38^{o}32´N, longitude 26^{o}34´E and almost 20 meters above from the sea and in the coast of the sea while Gemlik olive and its leaf samples were collected from the orchard of İYTE campus which is situated at latitude 38^{o}19´N, longitude 26^{o}37´E. Sample collections were done throughout two months by one week intervals. Samples were collected from at least three different trees during theirs maturation periods which began at the middle of October and ended at the beginning of December. Hurma olive maturation determined by starting of creasing and colour change to brown. Olive and leaf samples were picked by hand using sterile equipment. All the samples were collected into sterile jars and kept at +4^{o}C until they were analysed.

Moreover air samples were obtained from both of orchards by the methods of sedimentation; the petri dishes with convenient medium (plate count agar for bacteria, potato dextrose agar for yeasts-molds and violet red bile glucose agar for *Enterobacteriaceae*) were placed horizontally and exposed to the air for 5 min (Tuszynski and Satora, 2003). All the samples were collected for two subsequent -2011 and 2012- seasons as shown in the Table 3.1.

3.1.2 Media

All media were used listed in the appendix A.

3.1.3 Chemicals and Reagents

Chemical and reagents used in this study were listed in the appendix B.

3.1.4 Solutions

Solutions were itemized in appendix C.

Table 3.1 Sampling districts, origin and quantity of isolated samples

District	Origin	Number of Sampling		Number of Sampling Material
	Erkence	***2011***	***2012***	
Orchard of Eğlenhoca Village	Non-debittered olive	6	8	At least 3 different trees
	Debittered olive (Hurma)	8	8	At least 3 different trees
	Leaf	8	8	At least 3 different trees
	Air	8	8	2 petri dishes for each media
	Gemlik			
Orchard of İYTE Campus	Olive	7	8	At least 3 different trees
	Leaf	7	8	At least 3 different trees
	Air	7	8	2 petri dishes for each media

3.2. Methods

3.2.1. Microbiological Analysis of Cultivable Microbiota

For preparation of olive samples, olives were destoned aseptically. Then, 10 g of each olive and olive leaf samples were transferred in a pouch containing 90 ml of one of four strength Ringer's solution and then homogenized for 1 min in a stomacher. This homogeneous mixture was used as stock solution for preparing decimal dilution solutions. Serial dilutions of 10^{-1}, 10^{-2} and 10^{-3} were prepared using for each 9 ml of one

of four strength Ringer's solution. 0.1 mL aliquots of each samples' dilutions were spread onto Plate Count Agar (PCA) for enumeration of total aerobic mesophilic count after incubation at 30^{o} C for 48 hours; Violet Red Bile Glucose Agar (VRBGA) for enumeration of *Enterobacteriaceae* after incubation at 37^{o}C for 24 hours; and Baird Parker Agar supplemented with egg yolk tellurite emulsion (BPA) was used for enumeration of staphylococci and micrococci after incubation at 37^{o}C for 2 days. *Pseudomonas* was detected with Pseudomonas Agar base supplemented with SR 0102 E (PSA) after incubation at 25 ºC for 2 days. Potato Dextrose Agar containing 1% of tartaric acid (PDA), Oatmeal Agar (OA), Czapek-Dox Agar (CDA), Sabouraud Dextrose Agar (SDA) were used for enumeration molds and yeasts, and they were incubated at 30^{o}C for 3-5 days. For detection of lactic acid bacteria Lactobacilli MRS agar was poured as 1 ml aliquots of each samples and overlaid with the same agar medium, and incubated at 30 ^{o}C for 48 h in anaerobiosis (in Merck anaerobic jar) (Campaniello et. al., 2005, Panagou et. al., 2002, Aveskamp at. al.,2009).

3.2.2. Isolation of Bacteria

Representative monoculture colonies were randomly selected from the related agar media (VRBGA and PCA). Purification involved two consecutive steps: culturing in nutrient broth and streaking on nutrient agar until pure cultures were obtained when observed under light microscopy. Pure bacterial cultures were kept both in glycerol stocks at -80 ^{o}C and in nutrient agar slants at 4 ^{o}C.

3.2.3. Identification of Bacteria Isolated from Olive Fruits and Leaves

3.2.3.1. Morphological Identification of Bacteria

3.2.3.1.1 Microscopic Morphology

Bacteria isolates were classified according to their morphology of vegetative cells and spores using phase contrast microscope (Olympus-CX31, Japan). Isolates were grown on nutrient agar for 18-24 h at 37^{o}C. The examination slides were prepared by

suspension of isolates into 10µl of 0.8% of NaCl solution and covered by a lamel and examined under phase contrast microscope for their morphology and existence of spores.

3.2.3.1.2. Macroscopic Morphology

Isolates were purified by consecutive growth in nutrient broth followed by streaking on nutrient agar plates. Colony morphologies in terms of color, appearance of colony, surface texture, shape and size were examined by eye.

3.2.3.2. Gram Staining

The isolates were subdivided into groups according to their Gram reaction and their morphology for further identification analyses. Gram reaction of bacterial cells is based on their cell wall structure. To determine their Gram reaction, fresh cultures of isolates were used. Single colonies grown on Nutrient Agar plates were suspended into 15µl of 0.8% of NaCl solution onto examination slides and let them dry. Gram staining procedure was performed. The stained cultured were examined under light microscope (Olympus-CX31, Japan). Gram positive cells were purple while Gram negative cells remained pink or reddish.

3.2.3.3. Preservation of the Isolates

Isolates were transferred in nutrient broth containing 20% of gylcerol (v/v) as frozen stocks which were prepared by adding 500µl of overnight cultures in nutrient broth into 500 µl of 40% glycerol and stored at -80^0C. Gram staining procedure was followed.

3.2.4 Biochemical Tests of Identification

Biochemical (physical) tests to identify bacteria involve substantial subjective interpretations. No accurate results generally are obtained (Tang et. al., 1998). Diagnostic table and dichotomous keys are used to identify present the types of characters to be tested (Truper and Schleifer, 2006). In this study, biochemical tests were performed to divide the bacteria into subgroups. For this purpose, the following identification chart key was used for biochemical tests (Figure 3.1, Figure 3.2 and Figure 3.3) and all tests were not performed for each bacteria since they were not required for all of them.

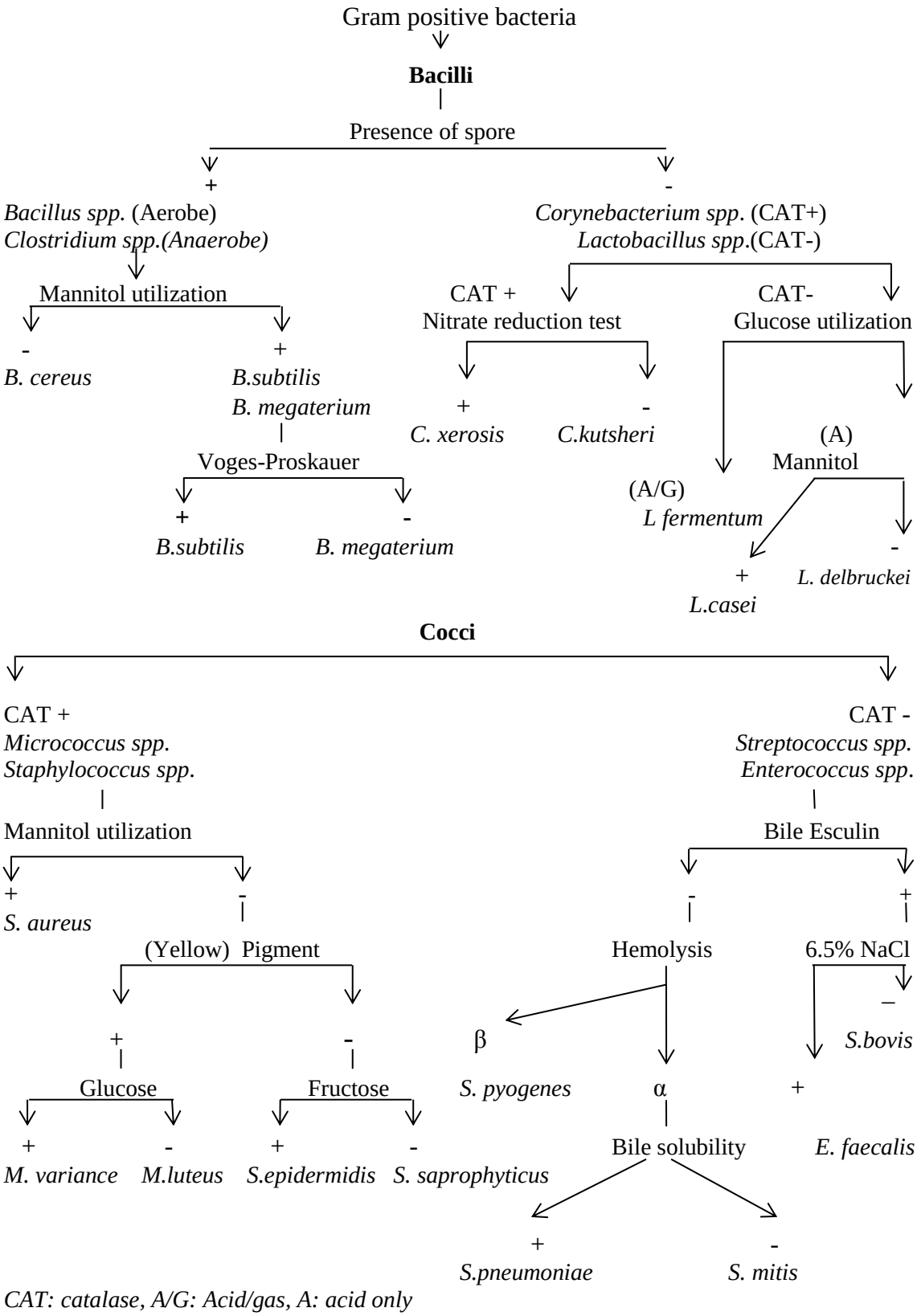

Figure 3.1 Identification flowchart for Gram positive bacteria
(Source: adapted from Buchanan and Gibbons, 1974)

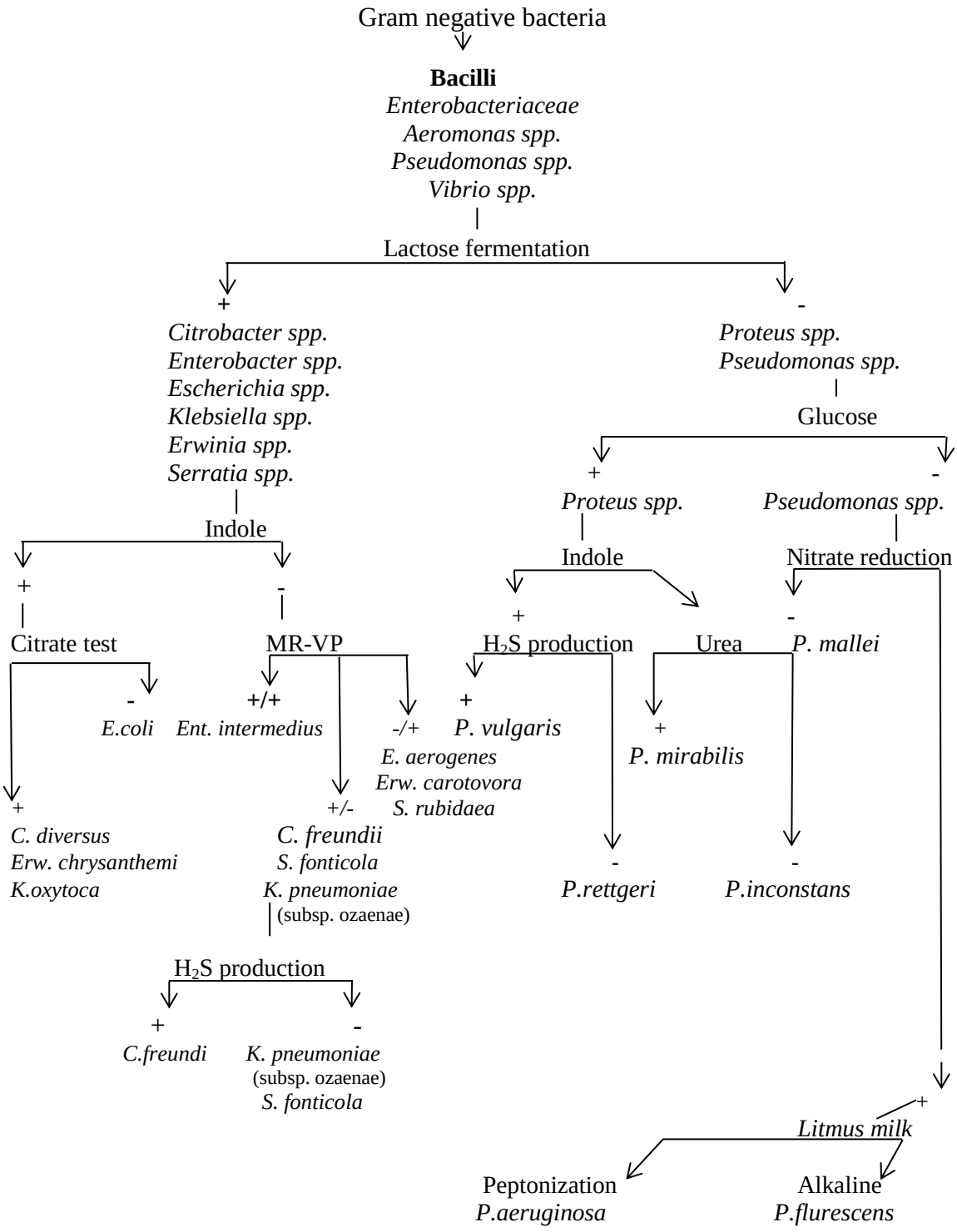

Figure 3.2 Identification flowchart for Gram negative bacilli bacteria
(Source: adapted from Buchanan and Gibbons, 1974)

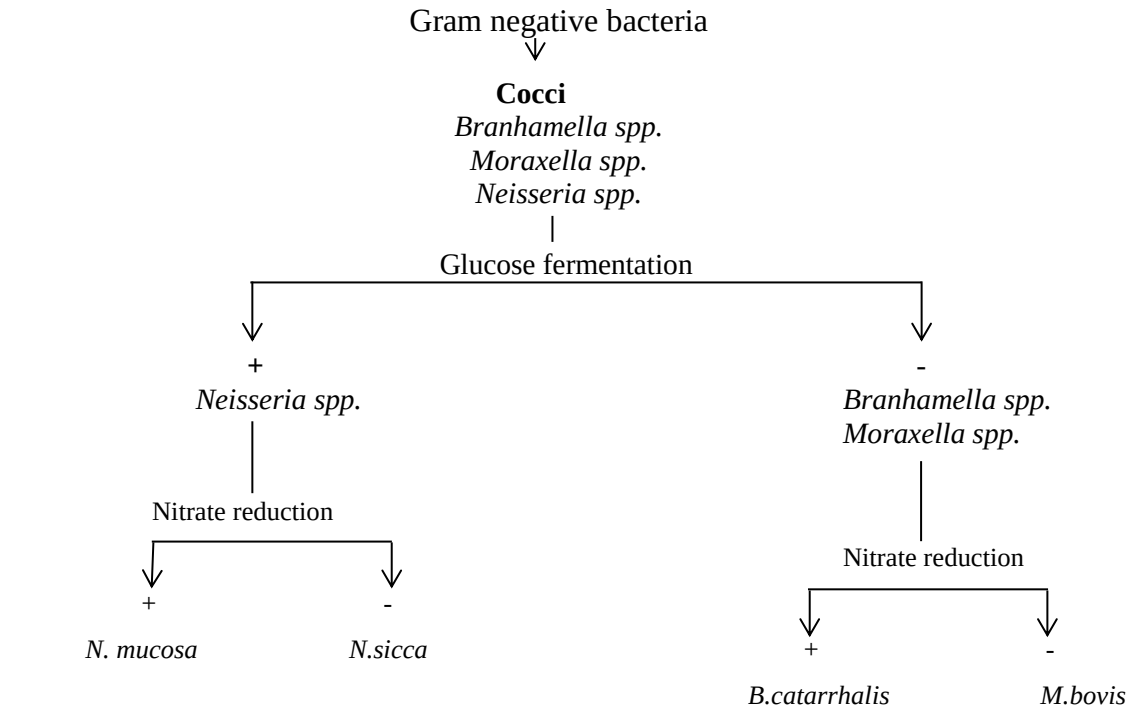

Figure 3.3 Identification flowchart for Gram negative cocci bacteria
(Source: adapted from Buchanan and Gibbons,1974)

3.2.4.1. Catalase Test

Catalase is an enzyme which catalyzes of the breakdown of hydrogen peroxide into oxygen and water. As a result of this reaction gas bubbles are formed. This is an important differentiation characteristic for bacteria.

$$2H_2O_2 \rightarrow O_2 + H_2O$$

In order to perform catalase test, isolates were grown on nutrient agar for 18-24 h at 37^o C and then 3% of H_2O_2 solution were dropped onto colonies. Formation of bubbles with water was the sign of positive result.

3.2.4.2. Carbohydrate Utilization Tests

Carbohydrate fermentation characteristics of isolates were determined according to seven different sugar compounds. For this purpose 96-well plates were used for analysis. 10 % of sugar solution (w/v) was prepared prior to activation of isolates. Isolates were activated in nutrient broth and incubated at 37^{o} C for 18-24 hours. After 1 ml of each isolates was transferred into eppendorf tubes they were centrifuged for 10 min at 10000 rpm. Afterwards, supernatant was removed and pellets were washed with 1mL PBS solution twice. Finally, after supernatant was discarded, pellets were resuspended in bromcresol purple nutrient broth. Sugar solutions were sterilized by filter (0.45µm pore diameter). After completion of preparation of sugar solutions and samples preparation, 40 µl of sugar solutions were pippeted into each wells and 160 µl of suspended active cultures were added onto sugar solutions. All sugar fermentation tests for each isolates repeated twice. Positive and negative control for comparison of isolates to test if any contamination has occurred or not. As positive control, 160 µl of *E. coli* suspension and 40 µl of glucose solution were used while 200 µl of *E. coli* suspension was used as negative control. 96-well plates were incubated overnight at 37^{o} C. Subsequently, turbidity and color change from purple to yellow was assumed as positive result. Besides, color between purple and yellow was assumed as weak positive results while purple remained wells were negative results as in Figure 4.2 For final step, results were compared with the absorbance of samples read at 620 nm in an automated microplate reader (Thermo Multiskan EX).

3.2.4.3. Voges-Proskauer (VP) Test

This test differentiates the bacteria according to their ability to ferment glucose. Pyruvic acid is the end products of glucose metabolism that is further metabolized by using butylene glycol pathway to produce neutral end such as acetoin and 2,3 butanediol. Main aim of this test is the detection of acetoin. Adding 5% solution of alpha naphthol and 40% KOH, detection of the presence of acetoin, which is the precursor in the 2,3- butanediol synthesis is possible.

Acetoin in the presence of oxygen is oxidized to diacetyl by alpha naphthol as as a catalyst. Afterwards, diacetyl reacts with guanidine components of peptone to form pinky red color component. When the reddish color is observed, the result of VP test is positive. Another reagent potassium hydroxide acts as an oxidizing agent and absorbs carbon dioxide present in the medium and accelerates the conversion of acetoin to diacetyl (Winn and Koneman, 2006). The VP test was done as below:

1. Isolates were inoculated in a tube of MR-VP broth which was grown for overnight on MacConkey Agar.

2. They were incubated for 24 hours at 37°C.

3. Before the use of the reagents, they were allowed to ward to room temperature.

4. 9 drops (0.6 ml) of α-naphthol reagent was added into each tubes and tubes were shaken gently.

5. 3 drops (0.2 ml) of 40% Potassium Hydroxide was added.

6. Tube was shaken gently for 30 seconds. The broth must be exposed to oxygen for a color reaction to occur.

7. Tube was allowed to stand for 15 minutes before evaluation.

8. If reddish color formed in the medium this was assumed as positive result.

3.2.4.4. Citrate Test

Citrate test is applied to differentiate the Gram negative bacteria according to the citrate utilization as a carbon source. The bacteria use the citrate in their Kreb's cycle. As a consequence of this metabolization, citrate is converted into oxalo acetic acid by the activity of citrate lyase. Oxaloacetate is further broken down to pyruvate and carbondioxide is released by the activity of oxaloacetate decarboxylase. Carbon dioxide reacts with ammonium dihydrogen phosphate and utilization of sodium citrate causes to an increase in the pH. In alkaline pH, bromthymole blue color reagent present in the media leads to color change from green to blue. After 18-24 h of incubation at 37^{o} C, reaction was observed. Blue color change indicated the citrate positive bacteria while citrate negative bacteria lead to no change in green color of media.

3.2.4.5. Urea Test

Urea test is used to differentiate the organisms that have the ability of production of urease enzyme. By dint of this enzyme present in the organism, the urea is broken down to carbon dioxide and ammonia.

$$(NH_2)_2CO + 2H_2O \xrightarrow{\text{Urease}} CO_2 + H_2O + 2NH_3$$

The presence of ammonia in the environment of medium increases the pH and alkalinity. Since the medium contains phenol red as a pH indicator, orange color of urea broth inoculated with urease positive organism turns to pink as a sign of positive reaction after incubation at 37^o C for 18-24 h. No color change is evidence of a negative reaction.

3.2.4.6 H_2S Production Test

Hydrogen sulfide production test is designated to differentiate the bacteria belong to family of *Enterobacteriaceae.* This test was conducted with Triple sugar iron agar slants. Principle of differentiation is based on the ability of carbohydrate fermentation patterns and H_2S production. Carbohydrate fermentation is accompanied by gas formation (bubble in agar, cracks and color change in agar due to pH decrease (from red to yellow) and black color due to H_2S production (black color).

3.2.4.7. Motility Test

This test differentiates the bacteria based on their ability of motility. For this purpose, semi-solid motility medium was inoculated with a needle along a line into butt and incubated for 24 h at 37^o C. Horizontally spread growth from inoculation line means that the organism is motile. Non-motile organisms' growth only occurs along the line of inoculation only.

3.2.4.8. Nitrate Reduction Test

The basis of nitrate reduction test is the determination of the ability of bacteria to reduce nitrate to nitrite or nitrogenous gases. The reduction of nitrate can be coupled to anaerobic respiration in some bacterial species due to the enzyme of nitrate reductase which is activated under anaerobic condition. When nitrate is reduced to nitrite which can be also reduced to further a component, adding the sulfanilic acid reagent leads to reaction with nitrite and red color components are formed. Secondly, reagent of α-naphthylamine is added onto complex of nitrite-sulfanilic acid to give a red precipitate. This means that the organism is positive for nitrate reduction test. But, at this point, if the red color components are not formed, the reaction is continued by addition of zinc powder. After adding zinc powder if the red color is observed it means that there is unreduced nitrate in the medium, so the result is negative (Winn and Koneman, 2006). The nitrate reduction test is performed as followed:

1. To obtain anaerobic condition, isolates were inoculated into a low surface area to depth ratio medium including inverted tubes to limits oxygen diffusion from overnight grown colonies.

2. Incubated into nitrate broths with inverted tubes for 18-24 hours at 37 ^{o}C. Incubation can last till 42-48 h to see growth.

3. When the gas formation occurred in the inverted tubes, the results were accepted as negative since the nitrate had been reduced to nitrogenous gases.

4. If there is no gas formation in inverted tubes, firstly 10 drops of sulfanilic acid were added into the tubes and then 10 drops of α-naphthylamine solution were added also.

5. Red color formation within 2 minutes were interpreted as positive.

6. When there is no color formation, 20 mg of Zinc powder was added.

7. The red color formation was accepted as negative for the test result since unreduced nitrate was present in the medium.

8. No color change in 5-10 minutes was accepted as positive result due to degradation of nitrate to further components.

3.2.4.9. Indole Test

Indole test is one of the biochemical identification procedures for organisms. Indole test determines the presence of the ezyme of tryptophanase in the bacteria. The organisms having tryptophanase enzyme activity can metabolize the amino acid tryptophan to indole, ammonia and pyruvic acid. When indole present in the tryptophane test medium, adding *p*-dimethylaminobenzaldehyde (Kovac's of Ehrlich's reagents) leads to development of red color on the surface of the medium and this indicates the positive reaction while no colour change indicates negative reaction because of lack of tryptophanase enzyme (Winn and Koneman, 2006). The reaction is performed as following:

1. The cultures were incubated into Tryptone water at 37 ^{o}C for 24 hours.
2. After incubation, 10-12 drops of Kovac's reagent added into each test tubes and shooke gently.
3. Red color formation was observed within 3-5 minutes.

3.2.4.10. Sucrose Utilization Test

A loopfull of previously grown cultures were streaked onto bromcresol purple sucrose nutrient agar containing 10% of sucrose. At the end of the 24 hours incubation at 37 ^{o}C sucrose positive cultures were turned the color of agar purple to yellow, while there was no color change for sucrose negative cultures.

3.2.5. Molecular Identification of Bacteria

3.2.5.1. Isolation of Genomic DNA

Genomic DNA was extracted from the cultures by using the method previously established by Cardinal et al., (1997). Solution used during DNA extraction were given in Appendix D. Procedure is as following:

1. Isolates are grown in nutrient broths for overnight.

2. Harvesting of cells by centrifugation for 2 min at 13000 g and 4^{o} C.

3. Removal of liquid phase, washing pellet with 500 µl 1xTE buffer (pH8) and centrifugation for 2 min at 13000 g and 4^{o} C.

3. Suspending into 200 µl 1xTE buffer (pH8) containing 25 % of sucrose and 30 mg/ml lysozyme.

4. Incubation for 1 hour at 37^{o} C in water bath.

5. Addition of 370 µl 1xTE buffer (pH8) containing Proteinase K (1mg/ml) and 30 µl 10% SDS, respectively.

6. Incubation for 1 hour at 37^{o} C in water bath.

7. Lysing of cells by the addition of 100 µl 5M NaCl and 80 µl CTAB/NaCl solution (10% of cetytrimethylammonium bromide, 0,7M NaCl) respectively

8. Incubation of lysed samples for 10 min at 65^{o} C in water bath.

9. Extraction with 750 µl chloroform/isoamylalcohol (24:1)

10. Centrifugation for 2 min at 13000 g and 4^{o} C.

11. Transferring of upper aqueous phase into a new eppendorf tube.

12. Second extraction with chloroform/isoamyl alcohol and centrifugation for 2 min at 13000 g.

13. Second transffering of the upper aqueous phase into a new eppendorf tube.

14. Precipitating of DNA by the addition of 500 µl 2-propanol.

15. If DNA wool is obtained, transfer the wool into a new eppendorf tube containing 500 µl 70% ethanol for washing.

16. If DNA wool is not observed, centrifugation for 10 min at 6000 rpm.

17. Addition of 500 µl 70% ethanol with centrifugation at 6.000 rpm for 10 min.

18. Removing the ethanol and drying the pellet at 37°C for 10 min in a oven.

19. Dissolving dried pellet in 100 µl 1xTE buffer (pH 8) containing 100µg/ml RNase.

20. Incubation for 1 hour at 37 ^{o}C in water bath.

21. Adjusting the samples 100 µl with 1xTE.

22. Applying of alternating heat shock twice to dissolve DNA (80 °C for 10 min, and -20 °C for 20 min).

23. Storage of dissolved genomic DNA samples at -20°C for long time if required. At the end of the procedure, isolation of genomic DNAs was controlled by a spectrophotometer (Nano Drop 8000, Thermo Scientific, Wilmington, USA).

3.2.5.2. Amplification of 16S rDNA Spacer Region by Polymerase Chain Reaction (PCR)

Amplification PCR were performed in 25 µl PCR mixture including 5 µl of genomic DNA as a template, 10 pmol of forward primer, 10 pmol of reverse primers, 2.5µl of 2 µM dNTPs, 2.5 µl of Mg free Taq polymerase buffer (Fermentas), 1.5 µl of 25mM $MgCl_2$ (Fermentas) and 1U Taq polymerase (Fermentas). Amplification of genomic DNA was performed in a BIO-RAD C1000 thermal cycler (France) with the following PCR conditions.

1. 94^o C for 5 min
2. 94^o C for 1 min in denaturation
3. 56^o C for 1 min in annealing
4. 72^o C for 1 min in elongation

(2–4) 40 cycles

5. 72^o C for 10 min in final extension

For the amplification of 16S rDNA region of isolates, two pairs of primers were used: EGE1 forward primer, EGE2 reverse primer and 341F forward, 518R reverse primers.

Forward Primer: EGE1: 5'-AGAGTTTGATCCTGGCTCAG-3' (Çınar, 2005)
Reverse Primer: EGE2: 5'CTACGGCTACCTTGTTACGA-3' (Yavuzdurmaz, 2007)
Forward Primer : 341F: 5'-CCTACGGGAGGCAGCAG-3' (Bahuriddin et al., 2009)
Reverse Primer 518R: 5'-ATTACCGCGGCTGCTGG-3' (Bahuriddin et al., 2009)

3.2.5.3. Electrophoresis of Amplified PCR Products

In order to control whether genomic DNA were amplified, electrophoresis was performed in a 1% of agarose gel including ethidium bromide. After cooling to 45 ^{o}C, 50 µl ethidium bromide solution (10mg/ml) was added. The agarose gel was poured into the gel casting stand and combs were placed. When the agarose gel was solidified,

the combs were removed and the stand was placed into the tank that was filled with 1X TAE buffer.

For loading the samples, 3 µl of 6X loading dye (Fermentas) was mixed with 10 µl of PCR product and loaded into each well.When the all samples were loaded, electrophoresis was run by 80 mV. PCR products were visualised under UV illuminator and documented by gel documentation system (Vilber Lourmat, France)

3.2.5.4. Purification of PCR Products

PCR product samples had to be purified before performing sequence analysis. For this purpose, Sephadex G-50 and spin receiver columns were used. 1g of Sephadex G-50 was dissolved into deionized water and mixed vigorously for five minutes. 650 µl of Sephadex G-50 was added into spin columns and waited around 30 mins. Colums stopper were were discarded and centrifuged at 4800 rpm for 2 minutes. Supernatant were removed and Sephadex spin columns were placed into eppendorfs to collect samples. 10-15 µl of PCR product was poured into the middle of the spin column containing Sephadex G-50. After centrifugation at 4800 rpm for 2 minutes, purified PCR product was collected into the sample storage tube. Purified samples with sephadex spin columns were measured by a spectrophotometer (Nano Drop 8000, Thermo Scientific, Wilmington, USA). Samples having 260/280 absorbance ratio between 1.8 and 2.0 were suitable for DNA sequence analysis.

3.2.5.5. Cycle Sequencing

Cycle sequence was performed to label nucleotides by fluorescence in a thermocycler. The most important difference from PCR is that the using of only one primer in each cycle sequencing reaction, thereby amplification product forms in a linear form not exponential. The other difference is that dideoxynucleotides are used which interrupts the extension of the DNA strand when incorporated.

For that purpose, PCR products were sequenced in forward direction using 341F primer and BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems), according to manufacturer's recommendations. Each sample including the following

mixture was prepared for thermocycler (BIO-RAD, C1000 Thermal Cycler, France) performing PCR for cycle sequencing:

DNA of each sample : 2 µl (3-10 ng/ µl)

Forward primer: 1µl (3.2pmol/ µl)

Sequencing Buffer: 1 µl BigDye Terminator v1.1,v3.1 5X buffer

BigDye: 2 µl (3.2 pmole/ µl)

Deionized water: 4 µl

Cycle sequencing conditions were shown below:

1. 96^{o} C for 1 min
2. 96^{o} C for 10 sec.
3. 50^{o} C for 5 sec
4. 60^{o} C for 4 min

} 30 cycles

5. 4^{o} C, forever

3.2.5.6. Sequencing of Amplified Cycle Sequence Products

When the cycle sequencing was completed, samples were purified in Sephadex G-50 columns again as mentioned previously. 10 µl of cycle sequenced and purified samples were placed into each well of microplate of sequence device (3130*xl* Genetic Analyzer, Applied Biosystems, California, USA) whose systems are based on the capillary electrophoresis. 7 ml of flowable polymer (3130 POP-7 Polymer) and 25 ml of buffer for anode and cathode poles (Genetic Analyzer 10X running buffer with EDTA) were loaded into the capillaries prior to run. After analysis were completed, nucleotide sequence results of cultures were evaluated by a software (Finch TV v.1.4.0, Geospiza.Inc) and the software searched sequence similarity of cultures by Basic Local Alignment Search Tool (BLAST) server via a web interface at http://www.ncbi.nlm.nih.gov/BLAST/.

3.2.6. Statistical Analysis

In each sampling time, mean values of microbial load of each samples and their standard deviation were calculated. Analyses of variance was performed to investigate the difference ($P<0.05$) in both season's enumeration results during maturation of olives. Tukey HSD test was also performed to compare mean values of enumeration results of for each medium and mean values of enumeration results of each sampling time between media. SPSS (version 16.0; SPSS Institute Inc., Chicago, IL) was used for all statistical analysis.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Microbial Load of Olive Fruits, Leaves and Orchard's Air

Throughout the maturation period 2011 and 2012 harvest years, microbiological analyses were performed with collected olive samples of Hurma and Erkence olives, Gemlik variety, their leaves and air from both olive orchards. In the further sections of this theis naturally debittered Erkence olive is referred to as 'Hurma' olive.

4.1.1. Microbial Profile of Erkence Olive Variety

4.1.1.1. Microbial Profile of Debittered Erkence -Hurma- Olive Variety

Change in microbial population change of total aerobic mesophilic count of hurma olive for two seasons are shown in Table 4.1. On the onset of maturation, initial count results of 2011 and 2012 ($\log_{10}$ 3.77 cfu g^{-1} and $\log_{10}$ 2.20 cfu g^{-1}, respectively) are close to each other. Ongoing maturation results followed an up and down line and finally peaked at $\log_{10}$ 6.89 cfu g^{-1} in the season of 2011. Increment in microbial load in the last weeks of maturation period is an expected result since oleuropein content decreases as olive maturates (Dağdelen et al., 2013). Besides, the conducted study showed that oleuropein level in self-debittered Dhokar variety olive which is similar to Hurma olive, declined and reached to a negligible level (0.06 g/kg) in fully ripened olive fruit (Jemai et al,2009). But there was a marked difference between two sesons' total aerobic mesophilics counts; in the second season the count of total aerobic mesophilic were lower than in 2011. This situation may be associated with lack of *Enterobacteriaceae* in 2012; while existence of *Enterobacteriaceae* in high numbers contributed to higher total aerobic mesophilic count in 2011. Total aerobic mesophilic count ranged between $\log_{10}$ 2.73 cfu g^{-1} and $\log_{10}$ 5.00 cfu g^{-1}; it was termined with $\log_{10}$ 4.16 cfu g^{-1} in 2012 which was 2.5 log unit lower than previous final count of total

aerobic mesophilic. Therefore, it could have been affected by climatic variables and alternate bearing in olive production (Fornaciari et al., 2002).

Yeasts and mold growth were monitored on various media to recover the widest range of fungi. Initially, in each sampling time of 2011 the counts from four different media (PDA,OA,SDA,CZA) were similar to each other with an exception in the first week as marked with subscripts (Table 4.1.) in which PDA and SDA were significantly differed with the highest and the lowest level of fungi load, respectively. In the rest of the sampling period, consistent enumerations were observed for microorganisms grown on four different media in each sampling time. The final counts of them were almost as high as the total aerobic mesophilic count in 2011. In other respect, in 2012 yeast and mold were enumerated in the range between $\log_{10}$ 3.15 cfu g^{-1} and $\log_{10}$ 5.88 cfu g^{-1}. The enumeration on PDA and SDA during maturation did not followed a stable trend while no significant differences of count results from OA and CZA were observed between sampling time as indicated with superscript in Table 4.1. In addition, no significant differences were observed between counts of yeast-mold grown on various agar media in each sampling time except 6^{th} week.

Enterobacteriaceae reached to the highest population ($\log_{10}$ 4.95 cfu g^{-1}) at the end of the sampling period in 2011, but in the following season they were at undetectable level until fifth week. Then population of them ranged between $\log_{10}$ 2.30 and $\log_{10}$ 3.67 cfu g^{-1}. The reason of undetected *Enterobacteriaceae* may be associated with inhibition of pathogenic bacteria such as *Stapyhylococcus aureus*, *Salmonella enteritidis*, germination of spores of *Bacillus cereus*, *Escherichia coli* by the high amount of phenolic compounds as stated in the study of Omar (2010). However, in a study of Aktaş et al. (2013) phenol content of Hurma olive in 2012 were detected at a lower level than the previous harvest years. In addition, mold and yeast population in 2012 was more dense than those in 2011; during monitoring of total aerobic mesophilic population on PCA, *Enterobacteriaceae* could have been suppressed by densely populated yeast and mold.

On the other hand, lactobacilli, pseudomonads, staphylococci and micrococci were not detected during maturation period for both seasons. This is consistent partially with the previous findings in unprocessed black Gemlik and Edincik olives (Borçaklı et al. 1993) in which lactobacilli were not observed above the detection limit. Another study supporting our finding for staphylococci and total aerobic mesophilic count

belongs to Mantzouridou and Tsimidou (2011). In their study, no staphylococci were detected in raw Thassos variety olives having 0.89 a_w and 10% of moisture content and total aerobic mesophilic count was $\log_{10}$ 6.6 $cfug^{-1}$ as observed in the season 2011 in our study. Results of no detection of pseudomonads, lactic acid bacteria and staphylococci confirm previous literature data that was reported also no detection of mentioned microorganisms group in dry-salted Thassos olives by Panagou et al. (2002).

4.1.1.2. Microbial Profile of Non-Debittered Erkence Olive Variety

Erkence olives as stated previously in this study was the unripened, bitter and green raw olive fruits. They were analyzed microbiologically to observe the difference of microbiological growth profile from Hurma olive and change of microbial load during maturation were given in Table 4.2. In 2011, all the olives on the trees turned to the debittered black Hurma olives two weeks earlier than in 2012. As expected, all types of microbial counts of non-debittered Erkence olives were lower than Hurma olive samples since the higher content of phenolic compounds present in Erkence olive tissue (Aktaş et al.,2013). Similarly in literature, regular Chemlali olive variety was compared to naturally debitttered Dhokar olive variety in terms of phenolic compounds during ripening. In green maturation stage almost 2-fold higher oleuropein was observed in Chemlali olive variety than those in Dhokar variety olive fruits and level of oleuropein did not declined as much as in Dhokar variety olive (Jemai et al., 2009).

In 2011, total aerobic mesophilic counts were generally higher almost one log unit than the corresponding results in 2012 for the first half of the maturation. In the last week of maturation in 2011 and 2012 seasons total aerobic mesophilic count results were $\log_{10}$ 2.66 cfu g^{-1} and $\log_{10}$ 3.96 cfu g^{-1}, respectively. But the seasonal difference must be taking into consideration since sampling of unripened Erkence olives was finished two weeks earlier in 2011. No significant differences were observed in sequent counts of total aerobic mesophilics during maturation in both seasons as shown statistically in marked superscripts in Table 4.2. and *p*-values determined as 0.064 and 0.076 in 2011 and 2012, respectively (Table 4.11).

Yeasts and molds were lower in two seasons compared to Hurma olive. Growth on the four media were similar to each other in 2011 and ranged between $\log_{10}$ 2.49 and $\log_{10}$ 3.65cfu g^{-1}. As seen in the Table 4.2 there were no significant differences between

sampling times (weeks) and media used for counting in terms of growth and count results. However, in 2012, yeast and mold counts were ranged between $\log_{10}$ 2.15 and $\log_{10}$ 5.78cfu g^{-1}. They were mostly stable on three media that were PDA, SDA and CZA. But counts obtained from OA was the most significantly different ones in five sampling time. Microbial load increased in the last week of sampling as closing to the end of the maturation in 2012.

Enterobacteriaceae count of the first year was remained in a narrow range during the maturation period, averaging about $\log_{10}$ 2.4cfu g^{-1} of unripened Erkence olive. On contrary, in the second year no *Enterobacteriaceae* were detected above the detection limit (<100 cfu/g). This could be resulted from the densely populated yeast and mold that may have suppressed the growth of the *Enterobacteriaceae*. The rest of the investigated microorganims that were pseudomonads, staphylococci, micrococci and lactic acid bacteria count results were the same as found in all samples' and in accordance with the previous studies from literature (Mantzouridou and Tsimidou, 2011; Panagou, 2002; Borçaklı et al. 1993).

4.1.2. Microbial Profile of Gemlik Olive Variety

Gemlik olive variety was monitored by microbiological analyses and details of two seasons were given in Table 4.3. The microbial population of Gemlik olives generally was noticeably lower than Hurma olives. Initial numbers of total aerobic mesophilic counts were very close to each other for both seasons. In the first season it gradually increased except for the fifth week and then continued to increase up to $\log_{10}$ 4.69 cfu g^{-1}. Alike in 2012, first three weeks during maturation, gradual augmentation was observed, but in the fourth week almost one log unit reduction occurred that followed with a 2 log unit increment but in the last three weeks, it gradually decreased to $\log_{10}$ 2.57 cfu g^{-1}.

Similar to total aerobic mesophilic count , yeasts and molds counts' results were generally lower than Hurma olives. This was attributed to existence of phenolic compounds in Gemlik olive tissue opposite of Hurma olive. In 2011 yeasts and molds were ranged between $\log_{10}$ 2.15cfu g^{-1} and $\log_{10}$ 4.66 cfu g^{-1}. First five weeks higher counts were obtained from PDA; while in first tree weeks and in fifth week for the rest of the agar media (OA, SDA,CZA), yeast and molds counts were stable so no

differences between the counts from OA, SDA,CZA media in terms of growth of mold and yeast were observed in mentioned sampling times**.** The final population of yeasts and molds remained 2.5 log unit below the Hurma olives' population. This situation was related to higher phenolic content of Gemlik olives since phenolic compounds have a suppresion effect on the growth of microorganisms. Moreover, in 2012, the population of yeast-mold and total aerobic mesophilic were observed as having similar growth trend. The increment in the fifth week was remarkable. Then in the last periods of maturation, decrease was followed with an increase in the last week. Differences between media used in enumeration of yeast and mold was variable week by week as seen in Table 4.3.

Enterobacteriaceae was around $\log_{10}$ 2 cfu g^{-1} in the first year on the other hand, in 2012 they were absent during the whole the maturation.

Same results for lactobacilli, pseudomonads, staphylococci and micrococci was obtained in Gemlik olive as the other samples that were remained at undetectable level in both seasons. The findings belong to population of lactic acid bacteria and *Enterobacteriacea* (for 2012 season) are in accordance with the study of Kumral et al., (2013) in which growth of both microbial groups were not observed in Gemlik olives fruits. Also in the study of Borcakli et al. (1993) microbial composition of unprocessed ripe black Gemlik olives were investigated; in which no lactobacilli (<10 cfu/g) and coliform bacteria (<$1x10^4$ cfu/g) were detected above the detection limit similar to our findings. Moreover Borçaklı et al., (1993) studied about different microbial group in the same study, streptococci and anaerobic sulfide producing bacteria remained below the detection limit (<10 cfu/g) while Gram-negative bacteria ($3x10^5$ cfu/g) and yeast ($1x10^5$ cfu/g) were observed in high numbers. In addition total phenol contents of Gemlik olive in both harvest years were at a higher level compared to Hurma olive (Aktaş et al., 2013) that explains the lower microbial load of Gemlik olive for both harvest years than those in Hurma olive.

4.1.3. Microbial Profiles of Leaves of Erkence Olive Variety

Randomly collected leaf samples from the all sides of trees were analyzed. The enumeration results of Erkence leaf samples were presented in the Table 4.4. The

fluctuations in the nutritional and physical conditions of phyllosphere lead to variations in size of population (Lindow and Brandl, 2002).

In the first season, there was almost 2.5 log unit increment during maturation period in total aerobic mesophilic count and termined the maturation with $\log_{10}$ 6.39 cfu g^{-1}. On the other hand in the second season the increase rate was just 1 log unit and general count average was much lower than the first season. The final count of total aerobic mesophilic count was $\log_{10}$ 3.4 cfu g^{-1}.

The aforementined situation of discordant growth rate was also observed in the yeast-mold count results. In 2012, yeast-mold count did not reach up to high levels as in 2011. There was almost 2.5 decimal difference between two seasons enumeration results. Therefore, the great harmony for each sampling time was observed between results of total aerobic mesophilic counts and yeast-mold counts from related agar media in 2011 as marked with subscripts in Table 4.4. On the other hand, in 2011 during maturation no stable microbial population was observed that were significantly differed ($P<0.05$) week by week as noted in Table 4.11. In a study, microbial colonization on beet sugar leaves from different age was observed at different times of the year and concluded that in the warmest and driest months of the season bacterial diversity is at the lowest level while at the highest level in the cooler and rainy season (Lindow and Brandl, 2003). This situation supports the above findings about two years enumeration in leaf samples of Erkence olive variety. As seen in the Figure 4.1. and Table 4.8., maturation period in 2012 is warmer (higher temperature) and drier (low rainfall) than those in 2011.

Enterobacteriaceae population was detectable only in three weeks but in the rest of sampling period it remained below the detection limit. Moreover, no *Enterobacteriaceae* were detectable in the following season. The phenolic content of olive leaves can reach up to 60-90 mg/g; adverse effect of phenolic content present in olive leaves were shown against bacteria and fungi by in vitro studies (Omar, 2010 and Markin et al, 2002). In the study of Markin et al, (2002) it was presented that *Escherichia coli* cell, exposed to 0.6% (w/v) olive leaf extract were destructed completely. This result could be associated with our findings about lack of *Enterobacteriaceae* in the season of 2012.

Therefore no lactobacilli, pseudomonads, stapylococci or micrococci were detectable as the other samples.

4.1.4. Microbial Profiles of Leaves of Gemlik Olive Variety

Collection of Gemlik olive and leaf samples were started one week later than Erkence olive and leaf samples. In this respect, the second week's result of Erkence leaf count results correspond to the first week resuls of Gemlik variety leaf results (Table 4.5).

In both seasons total aerobic mesophilic counts were very close to each other but in the first season until fifth week, counts did not change; in the fifth week a high rise was observed then total aerobic mesophilic count gradually increased till the end of maturation and reached to $\log_{10}$ 6.18 cfu g^{-1}; while in 2012 total aerobic mesophilics count was less diversified. The highest total aerobic mesophilics count was noted in the samples of seventh week ($\log_{10}$ 3.95 cfu g^{-1}) and in the last week it was slightly decreased to $\log_{10}$ 3.78 cfu g^{-1}.

Yeasts and molds were gradually increased after second week in 2011 and reached up to $\log_{10}$ 6 cfu g^{-1}. On the other hand in 2012, it was followed up and down trend and did not increase as much as in 2011 and ended with a count around $\log_{10}$ 4 cfu g^{-1}. Same situation as mentioned above was observed; Gemlik olive orchards is situated in the close district to Eğlenhoca Village; so same weather contidions was effective in this region.

Enterobacteriaceae were nearly absent in the first half of maturation but in the last half of maturation they were slightly augmented to around $\log_{10}$ 2 cfu g^{-1}.

4.1.5. Microbial Flora of Erkence Cultivar's Orchard's Air

As stated in the study of Tuszynski and Satora (2003) limited nutrient availability of air prevents the growth and development of microorganisms; therefore, air microflora population generally is much lower in comparison with soil and fruits.

During maturation period in 2011, total aerobic mesophilic count of sedimented petri dishes by air of Erkence variety olive orchard were averaged around $\log_{10}$ 1.75 cfu/m^3 and in 2012, $\log_{10}$ 1.67 cfu/m^3. Yeasts and molds' counts were lower in comparison with total aerobic mesophilic counts. The average population counts were $\log_{10}$ 1.26 cfu/$m^3$and $\log_{10}$ 1.17 cfu/m^3, in 2011 and 2012, respectively.

The meteorological data (http://tumas.mgm.gov.tr) suggest that during maturation period, the highest temperature, the highest relative humidity, lower rainfall with shorter duration of insolation were in 2012. As stated previously in the study of Lindow and Brandl (2002) warmest and driest months of the season bacterial diversity is at the lowest level while it is at the highest level in the cooler and rainy seasons. In the study of Hasnain (1993), it was stated that high wind velocity decreases the concentration of some species of mold and their basidiospores. In addition to this rain decreases spores. According to these statements, when comparing two seasons' microbial load of air samples, a significant difference was obtained as seen in Table 4.9.; both total aerobic mesophilic count and yeast-mold count results in 2012 were lower than those in 2011 (Table 4.7) when lower rainfall and higher temperature were observed as shown in Table 4.8 and Figure 4.1.

Enterobacteriaceae were almost absent in 2011 except for three weeks when it remained at very low levels and no *Enterobacteriaceae* were cultivated from the air samples of Erkence orchard in 2012 (Table 4.6.).

4.1.6. Microbial Flora of Gemlik Cultivar's Orchard's Air

Gemlik olive orchard's air samples were analyzed microbiologically in two seasons; the detailed results were given in the Table 4.7. Total aerobic mesophilic count of air samples in first year were in similar level throughout the study period, averaging about $\log_{10}$ 1.73 cfu/m^3 showed no sifnificant differences between sampling time (Table 4.11). However, in 2012 the total aerobic mesophilic count results were less than the previous year's, averaging about $\log_{10}$ 1.2 cfu/m^3. It increased and remained stable for five week and followed by a decrease in the last two weeks of sampling.

Yeast and mold population of Gemlik orchards air samples were slightly lower than the corresponding total aerobic mesophilic counts for both harvest years although Tuszynski and Satora (2003) indicated that the crowded population in the atmosphere belongs to molds and yeasts. Molds-yeasts were enumerated as averages of $\log_{10}$ 1.4 cfu/m^3 and $\log_{10}$ 1.16 cfu/m^3, in 2011 and 2012, respectively. Except for one week, growth rate on PCA and PDA were parallel in each week without significant differences as marked with subscripts (Table 4.7).

Enterobacteriaceae counts in air samples of Gemlik orchard showed that in some weeks *Enterobacteriaceae* were absent, while they appeared and were counted after second week for three weeks and in the last week. Maximum population in 2011 was $\log_{10}$ 1.38 cfu/m^3. But no *Enterobacteriaceae* were monitored in 2012 during the study.

Table 4.1. Microbial load (log CFU/g) of the principal microbial groups found in Hurma olive for eight weeks of maturation periods

Microbial groups	**Time (days)**							
2011	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic	$3.77\pm0.26^{a}_{cd}$	$4.26\pm1.86^{a}_{a}$	$3.20\pm0.78^{a}_{b}$	$4.32\pm2.25^{a}_{a}$	$3.76\pm1.53^{a}_{a}$	$5.05\pm1.67^{a}_{a}$	$4.20\pm2.00^{a}_{a}$	$6.89\pm0.52^{a}_{a}$
Mold and yeast								
PDA-2011	$4.75\pm0.67^{a}_{d}$	$2.90\pm1.57^{a}_{a}$	$3.31\pm0.58^{a}_{b}$	$4.02\pm1.31^{a}_{a}$	$3.75\pm0.95^{a}_{a}$	$3.78\pm1.23^{a}_{a}$	$4.52\pm2.28^{a}_{a}$	$5.57\pm0.71^{a}_{a}$
OA-2011	$3.32\pm0.13^{ab}_{bc}$	$2.80\pm1.39^{a}_{a}$	$3.48\pm0.63^{ab}_{b}$	$3.87\pm1.02^{ab}_{a}$	$4.09\pm1.03^{ab}_{a}$	$4.32\pm1.44^{ab}_{a}$	$3.74\pm1.63^{ab}_{a}$	$6.30\pm1.42^{b}_{a}$
SDA-2011	$2.46\pm0.79^{a}_{b}$	$3.39\pm1.08^{ab}_{a}$	$3.70\pm0.67^{ab}_{b}$	$3.93\pm1.78^{ab}_{a}$	$3.62\pm1.44^{ab}_{a}$	$4.59\pm1.57^{ab}_{a}$	$4.68\pm2.35^{ab}_{a}$	$6.54\pm0.28^{b}_{a}$
CZA-2011	$3.26\pm0.05^{a}_{bc}$	$3.83\pm0.55^{a}_{a}$	$4.12\pm1.18^{a}_{b}$	$3.56\pm1.36^{a}_{a}$	$4.09\pm1.11^{a}_{a}$	$4.48\pm1.73^{a}_{a}$	$4.46\pm2.14^{a}_{a}$	$6.45\pm1.12^{a}_{a}$
Enterobacteriaceae	<100 cfu/g $^{a}_{a}$	$2.85\pm1.4^{ab}_{a}$	<100 cfu/g $^{a}_{a}$	$2.82\pm1.43^{ab}_{a}$	$3.29\pm1.47^{ab}_{a}$	$4.65\pm2.35^{ab}_{a}$	$3.11\pm1.93^{ab}_{a}$	$4.95\pm2.76^{b}_{a}$
Pseudomonads	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Staphylococci & Micrococci	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Lactobacilli	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
2012	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic	$3.30\pm1.30^{ab}_{b}$	$2.73\pm0.73^{a}_{b}$	$3.23\pm0.28^{ab}_{b}$	$4.88\pm0.19^{b}_{b}$	$4.68\pm0.68^{b}_{a}$	$3.22\pm0.22^{ab}_{ab}$	$5.00\pm0.52^{b}_{b}$	$4.16\pm0.26^{ab}_{b}$
Mold and yeast								
PDA-2012	$3.15\pm1.50^{a}_{b}$	$4.48\pm0.71^{bc}_{c}$	$3.39\pm0.09^{ab}_{b}$	$5.01\pm0.09^{c}_{b}$	$4.90\pm0.20^{bc}_{a}$	$4.23\pm0.46^{abc}_{bc}$	$4.75\pm0.27^{bc}_{b}$	$4.45\pm0.54^{abc}_{b}$
OA-2012	$3.52\pm1.52^{a}_{b}$	$5.07\pm0.44^{a}_{c}$	$3.73\pm0.03^{a}_{b}$	$4.82\pm0.34^{a}_{b}$	$4.88\pm0.28^{a}_{a}$	$4.95\pm0.34^{a}_{c}$	$4.68\pm0.38^{a}_{b}$	$4.12\pm0.34^{a}_{b}$
SDA-2012	$3.69\pm0.79^{ab}_{b}$	$4.40\pm0.08^{abc}_{c}$	$3.34\pm0.26^{a}_{b}$	$4.73\pm0.26^{abc}_{b}$	$4.49\pm1.21^{abc}_{a}$	$5.09\pm0.05^{bc}_{c}$	$5.88\pm0.19^{c}_{b}$	$4.21\pm0.26^{ab}_{b}$
CZA-2012	$4.03\pm0.43^{a}_{b}$	$4.07\pm0.47^{a}_{bc}$	$3.25\pm0.34^{a}_{b}$	$4.72\pm0.42^{a}_{b}$	$4.10\pm0.90^{a}_{a}$	$4.18\pm0.87^{a}_{bc}$	$4.95\pm0.95^{a}_{b}$	$4.32\pm0.15^{a}_{b}$
Enterobacteriaceae	<100 cfu/g $^{a}_{a}$	<100 cfu/g $^{a}_{a}$	<100 cfu/g $^{a}_{a}$	<100 cfu/g $^{a}_{a}$	$3.67\pm1.67^{b}_{a}$	$2.30\pm0.30^{b}_{a}$	$2.50\pm0.50^{b}_{a}$	$2.58\pm0.58^{b}_{a}$
Pseudomonads	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Staphylococci & Micrococci	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Lactobacilli	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g

Results expressed as mean±standars deviation. Means along a row (superscript) or coloumn (subscript) without common letters (a-d) are significantly different ($p<0.05$).

Table 4.2. Microbial load (logCFU/g) of the principal microbial groups found in non-debittered Erkence olive for eight weeks of maturation periods

Microbial groups	**Time (days)**							
2011	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic	$4.04\pm0.51^{a}_{a}$	$2.85\pm0.49^{a}_{b}$	$3.04\pm0.65^{a}_{a}$	$4.14\pm1.01^{a}_{a}$	$2.65\pm0.52^{a}_{ab}$	$2.66\pm0.77^{a}_{a}$		
Mold and yeast								
PDA-2011	$3.49\pm0.25^{a}_{a}$	$2.62\pm0.55^{a}_{b}$	$3.59\pm0.65^{a}_{a}$	$3.44\pm1.28^{a}_{a}$	$2.49\pm0.35^{a}_{ab}$	$2.96\pm0.69^{a}_{a}$		
OA-2011	$2.89\pm0.87^{a}_{a}$	$2.60\pm1.04^{a}_{b}$	$3.31\pm0.79^{a}_{a}$	$3.65\pm1.43^{a}_{a}$	$3.01\pm0.18^{a}_{ab}$	$3.37\pm0.40^{a}_{a}$		
SDA-2011	$3.52\pm0.11^{a}_{a}$	$3.49\pm0.43^{a}_{b}$	$3.47\pm0.42^{a}_{a}$	$3.80\pm1.57^{a}_{a}$	$3.39\pm0.39^{a}_{b}$	$2.79\pm0.25^{a}_{a}$		
CZA-2011	$2.49\pm0.85^{a}_{a}$	$2.83\pm0.75^{a}_{b}$	$3.38\pm0.36^{a}_{a}$	$4.11\pm1.89^{a}_{a}$	$3.05\pm0.16^{a}_{ab}$	$3.09\pm0.69^{a}_{a}$		
Enterobacteriaceae	$2.81\pm0.57^{b}_{a}$	<100 cfu/g $^{a}_{a}$	$2.63\pm1.92^{b}_{a}$	$3.20\pm1.83^{b}_{a}$	$2.35\pm0.39^{ab}_{a}$	$2.23\pm0.40^{ab}_{a}$		
Pseudomonads	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g		
Staphylococci & Micrococci	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g		
Lactobacilli	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g		
2012	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic	$2.20\pm0.34^{a}_{b}$	$2\pm0^{a}_{b}$	$2.15\pm0.21^{a}_{b}$	$2.73\pm0.36^{a}_{b}$	$3.37\pm0.36^{a}_{b}$	$2.65\pm0.91^{a}_{bc}$	$2.38\pm0.55^{a}_{b}$	$3.96\pm2.77^{a}_{bc}$
Mold and yeast								
PDA-2012	$2.86\pm1.02^{a}_{bc}$	$2.47\pm0.67^{a}_{b}$	$3.27\pm0.60^{a}_{b}$	$2.50\pm0.70^{a}_{b}$	$2.86\pm0.51^{a}_{b}$	$2.23\pm0.33^{a}_{bc}$	$3.59\pm0.73^{a}_{c}$	$2.62\pm0.21^{a}_{ab}$
OA-2012	$3.59\pm0.25^{abc}_{bc}$	$3.96\pm0.26^{bc}_{c}$	$4.62\pm0.46^{c}_{c}$	$4.40\pm0.21^{c}_{c}$	$2.65\pm0.75^{a}_{b}$	$3.08\pm0.55^{ab}_{c}$	$3.32\pm0.02^{ab}_{c}$	$5.78\pm0.59^{d}_{c}$
SDA-2012	$2.96\pm0.63^{ab}_{bc}$	$2.84\pm1.20^{ab}_{ab}$	$2.57\pm0.81^{ab}_{b}$	$2.15\pm0.21^{a}_{b}$	$2.87\pm0.92^{ab}_{b}$	$2.38\pm0.12^{a}_{bc}$	$3.56\pm0.07^{ab}_{c}$	$4.34\pm1.47^{b}_{bc}$
CZA-2012	$4.11\pm0.80^{ab}_{c}$	$2.88\pm0.83^{ab}_{ab}$	$2.84\pm1.20^{ab}_{b}$	$2.33\pm0.57^{a}_{b}$	$2.65\pm0.75^{ab}_{b}$	$2.15\pm0.21^{a}_{b}$	$2.83\pm0.28^{ab}_{bc}$	$4.72\pm1.42^{b}_{bc}$
Enterobacteriaceae	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$
Pseudomonads	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Staphylococci & Micrococci	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Lactobacilli	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g

Results expressed as mean±standars deviation. Means along a row (superscript) or coloumn (subscript) without common letters (a-c) are significantly different ($p<0.05$)

Table 4.3. Microbial load (log CFU/g) of the principal microbial groups found in Gemlik olive variety for eight weeks of maturation periods

Microbial groups	**Time (days)**							
2011	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic		$2.15\pm0.15^{a}{}_{a}$	$2.23\pm0.23^{ab}{}_{b}$	$2.80\pm0.19^{abc}{}_{b}$	$3.06\pm0.11^{bc}{}_{ab}$	$2.45\pm0.15^{abc}{}_{a}$	$3.15\pm0.15^{c}{}_{b}$	$4.69\pm0.08^{d}{}_{c}$
Mold and yeast								
PDA-2011		$4.25\pm0.04^{de}{}_{b}$	$3.58\pm0.11^{bc}{}_{b}$	$3.14\pm0.10^{ab}{}_{b}$	$4.45\pm0.05^{e}{}_{cd}$	$4.45\pm0.05^{e}{}_{cd}$	$4.06\pm0.11^{cde}{}_{cd}$	$3.95\pm0.04^{cd}{}_{b}$
OA-2011		$2.15\pm0.15^{a}{}_{a}$	$2.34\pm0.34^{a}{}_{b}$	$2.84\pm0.06^{ab}{}_{b}$	$4.18\pm0.07^{c}{}_{cd}$	$3.77\pm0.17^{bc}{}_{a}$	$3.76\pm0.16^{bc}{}_{bc}$	$4.06\pm0.11^{c}{}_{bc}$
SDA-2011		$2.23\pm0.23^{a}{}_{a}$	$2.38\pm0.38^{a}{}_{b}$	$2.91\pm0.03^{ab}{}_{b}$	$4.66\pm0.07^{c}{}_{d}$	$3.69\pm0.21^{bc}{}_{a}$	$4.70\pm0.11^{c}{}_{de}$	$3.92\pm0.07^{bc}{}_{b}$
CZA-2011		$2.30\pm0.30^{a}{}_{a}$	$2.34\pm0.34^{a}{}_{b}$	$3.01\pm0.06^{ab}{}_{b}$	$3.74\pm0.06^{bc}{}_{bc}$	$2.38\pm0,38^{a}{}_{a}$	$4.86\pm0.08^{c}{}_{e}$	$4.08\pm0.08^{bc}{}_{bc}$
Enterobacteriaceae		$2.15\pm0.15^{b}{}_{a}$	<100 cfu/g $^{a}{}_{a}$	<100 cfu/g $^{a}{}_{a}$	$2.30\pm0.30^{b}{}_{a}$	$3.30\pm0.60^{b}{}_{a}$	$2.15\pm0.15^{b}{}_{a}$	$2.23\pm0.23^{b}{}_{a}$
Pseudomonads		<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Staphylococci & Micrococci		<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Lactobacilli		<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
2012	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic	$2.23\pm0.23^{a}{}_{b}$	$2.80\pm0.10^{ab}{}_{b}$	$3.38\pm0.12^{ab}{}_{b}$	$2.47\pm0.47^{a}{}_{b}$	$4.28\pm0.41^{b}{}_{b}$	$3.45\pm0.15^{ab}{}_{bc}$	$2.57\pm0.27^{ab}{}_{b}$	$2.57\pm0.27^{ab}{}_{b}$
Mold and yeast								
PDA-2012	$3.80\pm0.10^{bc}{}_{c}$	$3.75\pm0.15^{bc}{}_{c}$	$4.19\pm0.11^{c}{}_{c}$	$2.45\pm0.45^{a}{}_{b}$	$5.28\pm0.03^{d}{}_{bc}$	$2.77\pm0.17^{ab}{}_{b}$	$2.82\pm0.12^{ab}{}_{b}$	$3.75\pm0.05^{bc}{}_{c}$
OA-2012	$2.83\pm0.13^{a}{}_{b}$	$3.94\pm0.03^{b}{}_{c}$	$4.64\pm0.10^{c}{}_{cd}$	$3.45\pm0.15^{ab}{}_{b}$	$5.51\pm0.14^{d}{}_{c}$	$3.75\pm0.15^{b}{}_{c}$	$3.07\pm0.07^{a}{}_{b}$	$4.91\pm0.06^{cd}{}_{d}$
SDA-2012	$5.02\pm0.02^{c}{}_{d}$	$3.88\pm0.11^{b}{}_{c}$	$3.08\pm0.08^{ab}{}_{b}$	$2.47\pm0.47^{a}{}_{b}$	$5.44\pm0.06^{c}{}_{c}$	$3.15\pm0.15^{ab}{}_{bc}$	$3.29\pm0.18^{ab}{}_{b}$	$3.31\pm0.03^{ab}{}_{bc}$
CZA-2012	$3.99\pm0.09^{bc}{}_{c}$	$2.97\pm0.02^{ab}{}_{b}$	$4.87\pm0.02^{cd}{}_{d}$	$2.50\pm0.50^{a}{}_{b}$	$5.47\pm0.06^{d}{}_{c}$	$3.82\pm0.12^{bc}{}_{c}$	$3.37\pm0.32^{ab}{}_{b}$	$3.97\pm0.02^{bc}{}_{cd}$
Enterobacteriaceae	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$
Pseudomonads	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Staphylococci & Micrococci	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Lactobacilli	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g

Results expressed as mean±standars deviation. Means along a row (superscript) or coloumn (subscript) without common letters (a-d) are significantly different ($p<0.05$).

Table 4.4. Microbial load (log CFU/g) of the principal microbial groups found in leaves of Erkence olive variety

Microbial groups	**Time (days)**							
2011	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic	$4.04\pm0.42^{ab}{}_{a}$	$3.69\pm0.52^{a}{}_{b}$	$4.97\pm2.06^{ab}{}_{b}$	$4.67\pm0.22^{ab}{}_{a}$	$4.03\pm0.66^{ab}{}_{a}$	$5.06\pm0.83^{ab}{}_{b}$	$5.50\pm1.06^{ab}{}_{b}$	$6.39\pm0.05^{b}{}_{b}$
Mold and yeast								
PDA-2011	$4.81\pm0.55^{cd}{}_{a}$	$2.85\pm0.20^{a}{}_{b}$	$2.99\pm0.87^{a}{}_{b}$	$3.13\pm0.72^{ab}{}_{a}$	$3.62\pm0.55^{abc}{}_{a}$	$4.17\pm0.74^{abc}{}_{b}$	$4.71\pm0.46^{bcd}{}_{b}$	$5.95\pm0.18^{d}{}_{b}$
OA-2011	$4.78\pm0.50^{ab}{}_{a}$	$3.38\pm0.08^{a}{}_{b}$	$3.91\pm0.98^{a}{}_{b}$	$4.81\pm0.43^{ab}{}_{a}$	$4.07\pm0.90^{a}{}_{a}$	$4.65\pm0.60^{ab}{}_{b}$	$4.92\pm0.47^{ab}{}_{b}$	$5.96\pm0.11^{b}{}_{b}$
SDA-2011	$4.42\pm0.25^{ab}{}_{a}$	$3.62\pm0.25^{a}{}_{b}$	$4.41\pm1.07^{ab}{}_{b}$	$4.75\pm0.31^{ab}{}_{a}$	$4.40\pm0.99^{ab}{}_{a}$	$4.60\pm1.11^{ab}{}_{b}$	$4.81\pm0.14^{ab}{}_{b}$	$5.99\pm0.10^{b}{}_{b}$
CZA-2011	$4.32\pm0.08^{ab}{}_{a}$	$3.14\pm0.66^{a}{}_{b}$	$4.11\pm0.85^{ab}{}_{b}$	$5.21\pm0.32^{b}{}_{a}$	$4.17\pm1.17^{ab}{}_{a}$	$4.68\pm1.00^{ab}{}_{b}$	$5.21\pm0.44^{b}{}_{b}$	$5.79\pm0.57^{b}{}_{b}$
Enterobacteriaceae	$3.77\pm0.26^{b}{}_{a}$	<100 cfu/g $^{a}{}_{a}$	<100 cfu/g $^{a}{}_{a}$	$3.02\pm1.76^{b}{}_{a}$	$2.39\pm0.67^{b}{}_{a}$	<100 cfu/g $^{a}{}_{a}$	<100 cfu/g $^{a}{}_{a}$	<100 cfu/g $^{a}{}_{a}$
Pseudomonads	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Staphylococci & Micrococci	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Lactobacilli	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
2012	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic	$2.40\pm0.34^{a}{}_{b}$	$3.79\pm0.50^{b}{}_{b}$	$3.63\pm0.53^{ab}{}_{bc}$	$3.54\pm0.28^{ab}{}_{b}$	$3.18\pm0.77^{ab}{}_{bc}$	$2.50\pm0.17^{ab}{}_{b}$	$3.21\pm0.40^{ab}{}_{b}$	$3.41\pm0.49^{ab}{}_{b}$
Mold and yeast								
PDA-2012	$2.63\pm0.35^{a}{}_{b}$	$3.47\pm0.05^{b}{}_{b}$	$2.94\pm0.55^{ab}{}_{b}$	$3.34\pm0.28^{ab}{}_{b}$	$2.84\pm0.15^{ab}{}_{b}$	$3.12\pm0.20^{ab}{}_{b}$	$3.39\pm0.15^{ab}{}_{b}$	$3.04\pm0.08^{ab}{}_{ab}$
OA-2012	$3.00\pm0.41^{a}{}_{b}$	$4.55\pm1.32^{a}{}_{b}$	$4.38\pm0.20^{a}{}_{c}$	$3.78\pm0.68^{a}{}_{b}$	$4.02\pm0.33^{a}{}_{c}$	$3.94\pm0.70^{a}{}_{b}$	$3.88\pm0.06^{a}{}_{b}$	$3.63\pm0.05^{ac}{}_{b}$
SDA-2012	$3.38\pm0.94^{a}{}_{b}$	$3.38\pm0.94^{a}{}_{b}$	$3.86\pm0.42^{a}{}_{bc}$	$3.86\pm0.42^{a}{}_{b}$	$3.35\pm0.42^{a}{}_{bc}$	$3.62\pm0.59^{a}{}_{b}$	$3.97\pm0.68^{a}{}_{b}$	$3.68\pm0.63^{a}{}_{b}$
CZA-2012	$3.10\pm0.45^{a}{}_{b}$	$4.49\pm0.41^{a}{}_{b}$	$3.71\pm0.66^{a}{}_{bc}$	$3.36\pm0.93^{a}{}_{b}$	$3.89\pm0.23^{a}{}_{bc}$	$3.68\pm1.05^{a}{}_{b}$	$4.12\pm0.21^{a}{}_{b}$	$3.35\pm0.59^{a}{}_{b}$
Enterobacteriaceae	<100 cfu/g $_{a}$	100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$
Pseudomonads	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Staphylococci & Micrococci	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Lactobacilli	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g

Results expressed as mean±standars deviation. Means along a row (superscript) or coloumn (subscript) without common letters (a-d) are significantly different ($p<0.05$)

Table 4.5. Microbial load (log CFU/g) of the principal microbial groups found in leaves of Gemlik olive variety

Microbial groups	**Time (days)**							
2011	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic		$2.80\pm0.10^{ab}{}_{a}$	$2.45\pm0.15^{a}{}_{b}$	$3.78\pm0.16^{bc}{}_{b}$	$2.55\pm0.55^{ab}{}_{a}$	$4.95\pm0.04^{cd}{}_{d}$	$5.33\pm0.05^{d}{}_{cd}$	$6.18\pm0.11^{d}{}_{b}$
Mold and yeast								
PDA-2011		$4.12\pm0.04^{bc}{}_{b}$	$2.34\pm0.34^{a}{}_{b}$	$2.53\pm0.11^{a}{}_{b}$	$3.46\pm0.08^{b}{}_{ab}$	$4.89\pm0.06^{c}{}_{cd}$	$4.93\pm0.06^{c}{}_{bc}$	$6.25\pm0.12^{d}{}_{b}$
OA-2011		$2.34\pm0.34^{a}{}_{a}$	$2.38\pm0.38^{a}{}_{b}$	$2.50\pm0.24^{a}{}_{b}$	$4.07\pm0.12^{b}{}_{b}$	$4.38\pm0.15^{bc}{}_{bc}$	$4.58\pm0.11^{bc}{}_{b}$	$5.52\pm0.18^{c}{}_{b}$
SDA-2011		$2.95\pm0.04^{ab}{}_{a}$	$2.34\pm0.34^{a}{}_{b}$	$2.71\pm0.12^{ab}{}_{b}$	$3.30\pm0.07^{b}{}_{ab}$	$4.81\pm0.04^{c}{}_{cd}$	$5.18\pm0.13^{c}{}_{cd}$	$6.26\pm0.12^{d}{}_{b}$
CZA-2011		$2.97\pm0.02^{ab}{}_{a}$	$2.53\pm0.23^{a}{}_{b}$	$2.98\pm0.54^{ab}{}_{b}$	$4.27\pm0.12^{bc}{}_{b}$	$3.92\pm0.07^{b}{}_{b}$	$5.48\pm0.03^{c}{}_{d}$	$5.58\pm0.11^{c}{}_{b}$
Enterobacteriaceae		$2.30\pm0.30^{b}{}_{a}$	<100 cfu/g $^{a}{}_{a}$	<100 cfu/g $^{a}{}_{a}$	$2.15\pm0.15^{b}{}_{a}$	$2.15\pm0.15^{b}{}_{a}$	$2.86\pm0.08^{b}{}_{a}$	$2.23\pm0.23^{b}{}_{a}$
Pseudomonads		<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Staphylococci & Micrococci		<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Lactobacilli		<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
2012	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic	$2.58\pm0.11^{a}{}_{b}$	$3.34\pm0.14^{ab}{}_{b}$	$2.77\pm0.17^{a}{}_{b}$	$2.92\pm0.15^{a}{}_{b}$	$3.23\pm0.23^{ab}{}_{b}$	$2.95\pm0.04^{a}{}_{b}$	$3.95\pm0.12^{b}{}_{b}$	$3.78\pm0.02^{b}{}_{b}$
Mold and yeast								
PDA-2012	$2.30\pm0.30^{a}{}_{b}$	$5.24\pm0.27^{d}{}_{c}$	$3.23\pm0.23^{abc}{}_{bc}$	$2.73\pm0.26^{a}{}_{b}$	$4.41\pm0.23^{cd}{}_{cd}$	$2.97\pm0.02^{ab}{}_{b}$	$4.55\pm0.06^{d}{}_{c}$	$4.19\pm0.19^{bcd}{}_{bc}$
OA-2012	$2.97\pm0.19^{ab}{}_{b}$	$4.49\pm0.13^{d}{}_{c}$	$2.69\pm0.21^{a}{}_{b}$	$3.45\pm0.15^{abc}{}_{b}$	$3.99\pm0.04^{cd}{}_{bc}$	$3.38\pm0.08^{abc}{}_{bc}$	$4.78\pm0.08^{d}{}_{c}$	$3.63\pm0.17^{bc}{}_{b}$
SDA-2012	$2.66\pm0.18^{a}{}_{b}$	$4.64\pm0.16^{d}{}_{c}$	$3.34\pm0.19^{ab}{}_{bc}$	$2.89\pm0.05^{a}{}_{b}$	$4.95\pm0.04^{d}{}_{d}$	$3.76\pm0.11^{bc}{}_{c}$	$4.76\pm0.11^{d}{}_{c}$	$4.26\pm0.12^{cd}{}_{bc}$
CZA-2012	$2.98\pm0.15^{a}{}_{b}$	$4.75\pm0.08^{c}{}_{c}$	$3.92\pm0.07^{b}{}_{c}$	$2.72\pm0.12^{a}{}_{b}$	$3.34\pm0.08^{ab}{}_{b}$	$3.71\pm0.18^{b}{}_{c}$	$4.68\pm0.08^{c}{}_{c}$	$4.59\pm0.09^{c}{}_{b}$
Enterobacteriaceae	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$	<100 cfu/g $_{a}$
Pseudomonads	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Staphylococci & Micrococci	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Lactobacilli	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g

Results expressed as mean±standars deviation. Means along a row (superscript) or coloumn (subscript) without common letters (a-d) are significantly different ($p<0.05$).

Table 4.6. Microbial load (log CFU/m^3) of the principal microbial groups found in microflora of orchard of Erkence olive variety

Microbial groups	**Time (days)**							
2011	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic	$2.39\pm0.12^{d}_{c}$	$1.57\pm0.03^{b}_{b}$	$1.84\pm0.23^{bc}_{c}$	$1.86\pm0.12^{bcd}_{c}$	$2.19\pm0.15^{dc}_{c}$	$1.92\pm0.15^{bcd}_{b}$	$1.71\pm0.02^{bc}_{c}$	$0.53\pm0.08^{a}_{b}$
Mold and yeast	$1.13\pm0.02^{ab}_{b}$	$2.17\pm0.20^{c}_{c}$	$1.20\pm0.07^{ab}_{b}$	$1.15\pm0.06^{ab}_{b}$	$1.50\pm0.05^{b}_{b}$	$1.00\pm0.14^{a}_{a}$	$1.06\pm0.15^{ab}_{b}$	$0.89\pm0.07^{a}_{c}$
Enterobacteriaceae	$0.38\pm0.12^{b}_{a}$	0^{a}_{a}	0^{a}_{a}	0^{a}_{a}	$0.53\pm0.08^{bc}_{a}$	$0.72\pm0.17^{c}_{a}$	0^{a}_{a}	0^{a}_{a}
2012	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic	$1.83\pm0.06^{bc}_{b}$	$1.99\pm0.13^{c}_{c}$	$1.44\pm0.19^{b}_{a}$	$2.19\pm0.02^{c}_{c}$	$2.04\pm0.04^{c}_{c}$	$1.35\pm0.10^{b}_{c}$	$0.45\pm0.21^{a}_{a}$	$2.09\pm0.10^{c}_{c}$
Mold and yeast	$1.06\pm0.51^{a}_{ab}$	$1.45\pm0.08^{a}_{b}$	$1.18\pm0.68^{a}_{a}$	$1.41\pm0.12^{a}_{b}$	$1.35\pm0.08^{a}_{b}$	$0.77\pm0.10^{a}_{b}$	$0.58\pm0.15^{a}_{a}$	$1.61\pm0.16^{a}_{b}$
Enterobacteriaceae	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}

Results expressed as mean±standars deviation. Means along a row (superscript) or coloumn (subscript) without common letters (a-d) are significantly different ($p<0.05$).

Table 4.7. Microbial load (log CFU/m^3) of the principal microbial groups found in microflora of orchard of Gemlik olive variety

Microbial groups	**Time (days)**							
2011	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic		$1.44\pm0.51^{a}_{a}$	$1.50\pm0.06^{a}_{b}$	$1.96\pm0.15^{a}_{b}$	$2.07\pm0.45^{a}_{a}$	$1.75\pm0.06^{a}_{b}$	$1.76\pm0.12^{a}_{c}$	$1.68\pm0.18^{a}_{b}$
Mold and yeast		$1.90\pm0.80^{a}_{a}$	$1.32\pm0.07^{a}_{b}$	$1.30\pm0.13^{a}_{ab}$	$1.58\pm0.05^{a}_{a}$	$1.45\pm0.05^{a}_{b}$	$1.06\pm0.15^{a}_{b}$	$1.25\pm0.06^{a}_{ab}$
Enterobacteriaceae		0^{a}_{a}	0^{a}_{a}	$0.94\pm0.23^{bc}_{a}$	$1.38\pm0.21^{c}_{a}$	$0.15\pm0.21^{a}_{a}$	0^{a}_{a}	$0.62\pm0.21^{ab}_{a}$
2012	***0***	***7***	***14***	***21***	***28***	***35***	***42***	***49***
Total aerobic mesophilic	$0.84\pm0.21^{ab}_{b}$	$1.65\pm0.08^{b}_{b}$	$1.82\pm0.07^{b}_{b}$	$1.70\pm0.06^{b}_{b}$	$1.55\pm0.10^{b}_{b}$	$1.14\pm0.04^{b}_{b}$	$0.50\pm0.28^{a}_{ab}$	$0.45\pm0.63^{a}_{a}$
Mold and yeast	$0.82\pm0.18^{a}_{b}$	$1.46\pm0.01^{bc}_{b}$	$1.70\pm0.19^{c}_{b}$	$1.54\pm0.11^{c}_{b}$	$1.20\pm0.17^{abc}_{b}$	$0.72\pm0.17^{a}_{b}$	$0.89\pm0.07^{a}_{b}$	$0.97\pm0.09^{ab}_{a}$
Enterobacteriaceae	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}	0_{a}

Results expressed as mean±standars deviation. Means along a row (superscript) or coloumn (subscript) without common letters (a-c) are significantly different ($p<0.05$).

Table 4.8. General meteorological condition during the sampling periods in two years (Source: http://tumas.mgm.gov.tr)

	Relative humidity (%)	**Temperature (^{o} C)**			**Total Rainfall (mm)**	**Speed of Wind (m/sec)**	**Duration of Insolation (h)**
		Max T^o C	Min T^o C	Mean			
2011							
October	63.16	24.3	12	18.45	3.36	1.22	7.74
November	60.67	16.4	10.2	13.12	0	1.38	6.77
December	74.43	17.7	6.2	12.22	4.87	1.22	4.36
2012							
October	67.96	24.8	18.1	22.3	0.13	1.12	7.48
November	68.88	23.5	12.2	17.6	0.67	1.40	5.25
December	72.20	20.5	6.8	12.5	3.0	1.29	3.15

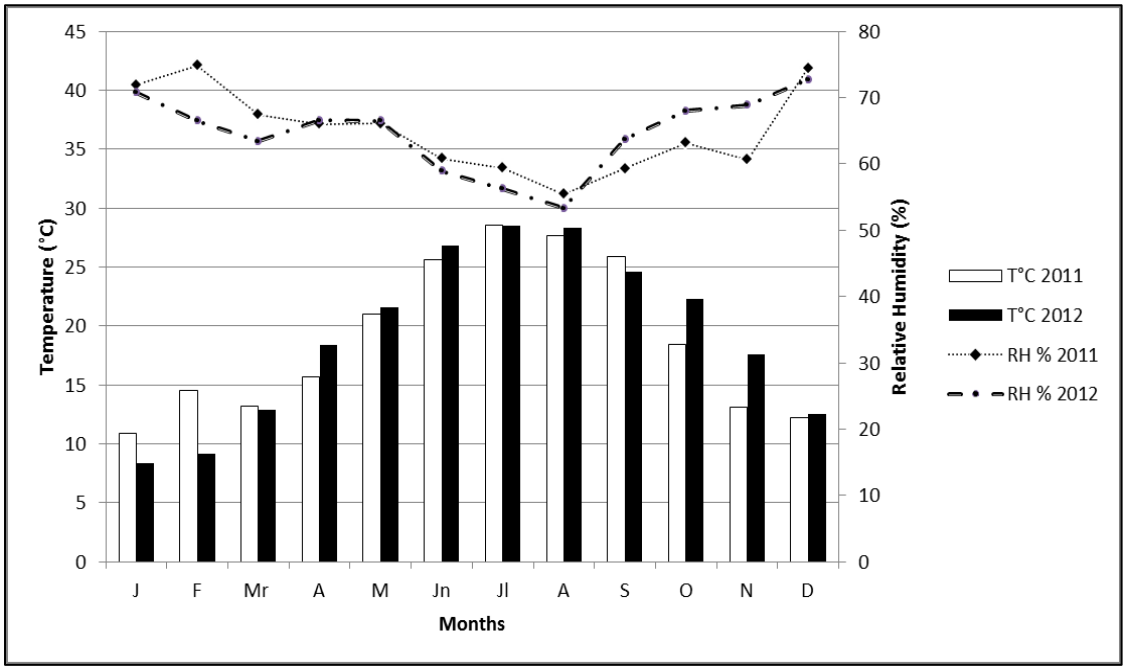

Figure 4.1. Average change in temperature and relative humidity in two years (Source: http://tumas.mgm.gov.tr)

4.2. Evaluation of Statistical Analyses

Analyses were carried out for each sample from both years. Analyses of variance (ANOVA) of the data for the population of microorganisms grown on various agar media in two years were performed (Table 4.9). For all samples, there were significant differences ($P<0.05$) in population of microorganisms grown on OA in both years. Differences of microbial profile obtained from various agar media for the samples of Gemlik variety leaves, Gemlik variety olives, air samples from Gemlik variety olive orchard, Erkence variety leaves and air samples of Erkence olive orchard were the most affected ones ($P<0.05$) while the effect of harvest year on Hurma olive was not significant (mostly $P>0.05$). Enumeration results obtained from media of PCA, OA and VRBGA were differed signicantly by year effect for all samples.

When Hurma olive was considered in respect to difference of enumeration between both years, it was observed that enumeration results significantly differed, except for PDA and CZA. Even though the climatic conditions may have contributed to differences in enumeration of microbial groups over the two-season, the marked differences in population of microbial groups can be explained by the fact of biennial bearing phenomena in olive production which is typical to olive.

Table 4.9. Probability values of the year effect on microorganisms grown on various agar media, calculated by ANOVA for each olive and leaf sample material.

Factor	Non-debittered Erkence Olive	Hurma Olive	Gemlik Olive	Erkence Olive Leaf	Gemlik Olive Leaf	Erkence Olive Orchard	Gemlik Olive Orchard
Sampling Seasons (2011 and 2012)							
PCA	0.001	0.026	0.000	0.000	0.000	0.000	0.000
PDA	0.206	0.078	0.000	0.000	0.000	0.002	0.004
OA	0.020	0.016	0.000	0.000	0.000		
SDA	0.122	0.009	0.000	0.000	0.001		
CZA	0.133	0.131	0.000	0.000	0.001		
VRBGA	0.000	0.000	0.000	0.000	0.000	0.000	0.000

In each time of sampling, it was expected to have parallel enumeration results on PCA and yeast&mold counting media; since the mentioned agar media are not selective except for VRBGA which is selective for the family *Enterobacteriaceae*. For this purpose, differences between enumerations with respect of media effect were analyzed for each sampling time in each season and their details were presented in the

Table 4.10. Generally, when there was no detection for *Enterobacteriaceae*, propability values of enumeration of bacterial groups was lower than 0.05 for media effects. As seen in the Table 4.10, in season of 2011, naturally bittered Erkence olive and non-debittered olive samples were the least affected samples from media effect since there were almost no significant differences between the counting results on various agar media. Similary, in results air samples from orchard of İYTE at 7th and 28th days of sampling did not differ significantly. Whereas, numbers for Gemlik olive, leaf samples of both olive varieties and air samples from Erkence olive orchard generally differed significantly by media effect. In 2012, since *Enterobacteriaceae* remained generally under detection limit, propability values of enumeration of bacterial groups for media effect were observed as lower than critical limit, so it was concluded that there were significant differences between media effects in most of the samples (Table 4.10).

Table 4.10. Probability values of enumeration of microbial groups between media effects for each sample material on sampling time, calculated by ANOVA for each samples.

Factor	Sampling time (days)							
	0	7	14	21	28	35	42	49
2011								
Hurma olive	0.000	0.716	0.000	0.889	0.971	0.965	0.934	0.582
Non-debittered Erkence olive	0.072	0.000	0.607	0.967	0.040	0.292		
Gemlik olive		0.001	0.002	0.000	0.000	0.076	0.000	0.000
Erkence leaf	0.037	0.000	0.002	0.024	0.126	0.000	0.000	0.000
Gemlik leaf		0.005	0.004	0.001	0.006	0.000	0.000	0.000
Air samples of Eğlenhoca	0.001	0.001	0.002	0.000	0.001	0.010	0.001	0.002
Air samples of İYTE		0.081	0.000	0.025	0.197	0.020	0.001	0.018
2012								
Hurma olive	0.024	0.000	0.000	0.000	0.603	0.000	0.000	0.001
Non-debittered Erkence olive	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Gemlik olive	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.000
Erkence leaf	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Gemlik leaf	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Air samples of Eğlenhoca	0.020	0.000	0.174	0.000	0.000	0.001	0.610	0.001
Air samples of İYTE	0.021	0.000	0.001	0.000	0.002	0.003	0.029	0.169

Maturation effect on microbial load of bacterial groups grown on related agar media for each year and sample materials were monitored and analyzed by one-way

ANOVA (Table 4.11). According to relevant table, total aerobic mesophilic counts and yeast-mold counts of Hurma, non-debittered Erkence olive and air samples of İYTE olive orchards were not changed significantly during maturation period of 2011. While significant differences were observed in whole enumeration results of Gemlik cultivars' olive fruit, Gemlik cultivars' leaves, Erkence cultivars' leaves and air samples of Erkence olive orchard in terms of maturation effects ($p<0.05$). Therefore, overall enumeration results of *Enterobacteriaceae* for each sampling material differed significantly during maturation period in 2011. In 2012, there were no significant differences in yeast-mold counts on PDA of non-debittered Erkence olive and on CZA of Hurma olive, air samples from Erkence olive orchard and total aerobic mesophilic count observed on PCA from the sample of non-debittered Erkence olive; also yeast-mold count did not differed significantly except for PDA in the leaves of Erkence cultivars in terms of maturation effects. Whereas the rest of the other samples presented in Table 4.11 showed significant maturation effect. On the other hand, microbial group originated from leaves and olive of Gemlik cultivars and air samples from Erkence olive orchards were the most affected by maturation effect. As stated in a study, different orchard management practices could be effective on phylloplane microorganism population (Bakker et al., 2002) which is attributed to explain the difference between results from both cultivars.

Table 4.11. Probability values of maturation effects on microbial groups (total aerobic mesophilic, yeast-mold and *Enterobacteriaceae*) among sample materials, calculated by ANOVA for each samples.

Factor	Total aerobic mesophilic count	Yeast and mold counts				*Enterobacteriaceae*
	PCA	PDA	OA	SDA	CZA	VRBGA
2011						
Hurma olive	0.197	0.307	0.078	0.088	0.191	0.017
Non-debittered Erkence olive	0.064	0.317	0.741	0.667	0.460	0.016
Gemlik olive	0.000	0.000	0.000	0.000	0.001	0.000
Erkence leaf	0.048	0.000	0.003	0.038	0.011	0.000
Gemlik leaf	0.000	0.000	0.000	0.000	0.000	0.000
Air samples of Eğlenhoca	0.000	0.000				0.000
Air samples of İYTE	0.376	0.310				0.000
2012						
Hurma olive	0.002	0.004	0.044	0.001	0.134	0.000
Non-debittered Erkence olive	0.076	0.108	0.000	0.020	0.010	
Gemlik olive	0.017	0.000	0.000	0.000	0.000	
Erkence leaf	0.001	0.023	0.166	0.381	0.234	
Gemlik leaf	0.017	0.000	0.000	0.000	0.000	
Air samples of Eğlenhoca	0.000	0.116				
Air samples of İYTE	0.003	0.001				

4.3. Isolation of Bacteria

Suspected bacterial colonies from PCA and VRBGA were isolated and purified (Panagou, 2002). 101 and 85 bacteria were isolated in the seasons of 2011 and 2012, respectively (Table 4.13). Samples comprised from Hurma olives (HO), Erkence olives (ERK), leaf of Erkence olive variety (HL) and Erkence olive orchard's air (EH). High amount of isolate belongs to season of 2011 since the means of microbial load counted on PCA and VRBGA were higher in whole sample materials than those in 2012 (Table 4.12).

Table 4.12. Means and standard deviations of microbial loads (log CFU/g) during maturation

	PCA		**VRBGA**	
	2011	2012	2011	2012
HO	4.43 ± 1.05	3.90 ± 0.82	2.70 ± 1.73	1.38 ± 1.43
ERK	3.23 ± 0.62	2.68 ± 0.63	2.20 ± 1.03	0 ± 0
HL	4.80 ± 0.83	3.21 ± 0.47	1.14 ± 1,52	0 ± 0
EH	1.75* ± 0.52	1.67* ± 0.54	0.17* ± 0.28	0* ± 0

*(logCFU/m^3)

4.4. Cultural Identification of Bacteria

4.4.1. Morphological Observation

Isolates were investigated under light microscopy to determine their morphological properties such as cell shape, size and arrangements. Overall, 160 bacilli and 26 cocci bacteria were isolated and purified from samples materials in 2011 and 2012 seasons.

4.4.2 Gram Staining and Catalase Test

Isolated pure bacterial cultures were grouped on the basis of Gram staining reaction and presence of catalase enzyme was determined by catalase test. It was found that all the bacteria isolates had catalase activity. Results based on Gram staining reaction with cell shapes was shown in the Table 4.13 and in details it can be seen in Table 4.14, Table 4.15 and Table 4.16. In 2011, amount of Gram negative bacilli isolates was higher than those in 2012, due to the high number of *Enterobacteriaceae* isolated from VRBGA. Conversly, more Gram positive bacilli were obtained in 2012. In addition, low amount of coccus shaped bacteria were isolated but no Gram negative cocci were observed in 2012 (Table 4.13).

Table 4.13. Distrubution of isolates according to their Gram reaction and cell shape

	2011				**2012**			
	Gram positive		Gram negative		Gram positive		Gram negative	
	Bacilli	Cocci	Bacilli	Cocci	Bacilli	Cocci	Bacilli	Cocci
HO	3		22	9	4		11	
ERK		1	12	2	4	2	2	
HL	12	9	8		18	1	19	
EH	13		8	2	21		3	
Total	**28**	**10**	**50**	**13**	**47**	**3**	**35**	
Gen.Total	**38**		**63**		**50**		**35**	

4.5. Results of Physiological and Biochemical Analyses

In this study biochemical tests were performed to divide bacteria into subgroups for further DNA sequencing analysis. For this aim, Gram positive bacilli, presence of spore, fermentative metabolism of carbohydrates, nitrate reduction and Voges-Proskauer test (Figure 4.2) were performed (Buchanan and Gibbons, 1974, Winn and Koneman, 2006). *Enterobacteriaceae* were identified according to previously given identification chart (Figure 3.2). Fermentative metabolism of carbohydrates especially lactose and glucose, tryptophanase activity by indole test, citrate utilization, MR-VP

test, H_2S production, urease activity and motility tests were performed. Besides, Gram positive cocci were identified according to their glucose, mannitol and fructose fermentation and yellow pigment production abilities. Especially, as the cultures of Gram-positive cocci got aged, pigment formation became more obvious.

Overall, the most isolated bacteria were Gram negative bacilli, Gram positive bacilli and Gram positive cocci in both years in descending order. All of the spore-forming aerobic bacilli were preidentified as belonging to genus of *Bacillus*. As reported in the literature (Campaniello et al., 2005), *Bacillus spp*. are prevailing among the mesophilic bacteria population in raw olive fruits. Mostly isolated Gram negative bacteria were observed as belonging to *Enterobacteriaceae* family which most of them isolated from VRBGA. Almost all of them were lactose and indole negative bacteria. The amount of cocci were much less than the bacilli in microflora of Hurma and in others'. Moreover , the most of cocci were observed in tetrad morphology and having yellow pigmented colonies. Most isolated Gram positive cocci bacterium was *Micrococcus luteus.* Detailed results of applied biochemical tests were given in the Table 4.14, Table 4.15 and Table 4.16. Not all of biochemical tests were performed for each sample; required tests were performed for each type of bacteria according to their physiological test results.

Figure 4.2. Gram staining and biochemical tests' results; **(**A) indole test, (B) nitrate test, (C) VP test, (D) carbohydrate test result on microplate, (E) sucrose utilization test, (F) citrate test, (G) motility test, (H) Gram-positive bacteria, (I) Gram-negative bacteria. (NEG: negative result, Pos: positive result)

Table 4.14. Physiological and biochemical test results of rod-shaped bacteria

Isolate name	Source	Gram Stain	Cell Morphgy	Spore	Catalase Activity	Carbohydrate fermentation								VP test	Indole Test	Nitrate test	ID based on DNA sequence analysis
						Lactose	Glucose	Fruktose	Arabinose	Mannitol	Maltose	Cellobiose	Sucrose				
2011																	
1-26	HO	+	Rod	+	+	-	+	+	+	+	-	-	-	+	-	+	*Bacillus simplex*
2-7	HO	-	Rod	-	+	-	-	-	-	-	-	-	-				
2-19	HO	-	Rod	-	+	+	+	+	+	+	+	+	+	+	+		*Klebsiella oxytoca*
4-13	HO	-	Rod	-	+	-	-	-	-	-	-	-	-		-		
4-14	HO	-	Rod	-	+	-	+	+	+	+	-	-	+		-		
4-15	HO	-	Rod	-	+	-	-	-	-	-	-	-	-		-		
5-10	HO	-	Rod	-	+	-	+	w	+	+	-	w	-		-	-	
5-13	HO	-	Rod	-	+	-	+	+	+	+	+	+	+		-	+	*Pantoea agglomerans*
6-2	HO	-	Rod	-	+	-	-	-	-	-	-	-	-		-		
6-4	HO	-	Rod	-	+	-	-	-	-	+	-	-	-		-		
6-7	HO	-	Rod	-	+	-	+	-	-	-	-	-	-		-		
6-10	HO	+	Rod	-	+	-	-	-	+	-	-	-	+		-		
7-13	HO	+	Rod	-	+	-	-	-	+	+	-	-	-	+	-	+	*Bacillus sp.*
8-16	HO	-	Rod	-	+	-	+	+	+	+	+	-	+		-		
8-17	HO	-	Rod	-	+	-	+	+	+	+	+	-	+	-	-	+	*Pseudomonas sp.*
8-20	HO	-	Rod	-	+	-	+	-	-	-	-	-	-	-	-	+	*Pseudomonas sp.*
2012																	
3-21	HO	+	Rod	+	+	-	-	-	+	+	-	-	+	+		+	
3-23	HO	+	Rod	+	+	-	-	-	+	+	-	-	-	+		+	*Bacillus subtilis*
5-1	HO	-	Rod	-	+	-	+	+	+	w	+	+	+		-	-	*Pantoea sp.*
5-2	HO	-	Rod	-	+	-	+	+	+	-	w	+	+		-	-	

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Table 4.14. (Cont.)

Isolate name	Source	Gram Stain	Cell Morphogy	Spore	Catalase Activity	Carbohydrate fermentation								VP test	Indole Test	Nitrate test	ID based on DNA sequence analysis
						Lactose	Glucose	Fruktose	Arabinose	Mannitol	Maltose	cellobiose	Sucrose				
5-3	HO	-	Rod	-	+	-	+	+	+	-	w	+	+		-	-	
6-15	HO	-	Rod	-	+	+	+	+	+	+	+	+	+	+	-	+	*Klebsiella pneumoniae*
7-1	HO	-	Rod	-	+	-	+	+	+	+	-	+	+	+	-		*Pantoea agglomerans*
7-2	HO	-	Rod	-	+	-	+	+	+	+	-	+	+	+	-		
8-24	HO	-	Rod	-	+	-	-	-	+	+	-	-	+	+	-		*Pantoea agglomerans*
8-25	HO	-	Rod	-	+	-	+	-	-	-	+	-	+	+	-		*Pantoea agglomerans*
2011																	
2-33	ERK	-	Rod	-	+	-	+	+	+	+	+	+	+	+	-	+	
2-35	ERK	-	Rod	-	+	-	+	+	+	+	+	+	+	+	-	+	*Pantoea agglomerans*
3-4	ERK	-	Rod	-	+	-	+	+	-	+	-	+	+		-	+	
4-6	ERK	-	Rod	-	+	-	+	+	-	-	-	-	+		-	+	
4-9	ERK	-	Rod	-	+	-	+	+	-	+	-	+	+		-	+	
4-11	ERK	-	Rod	-	+	-	+	+	-	+	-	+	+		-	+	
2012				-	+												
1-2	ERK	+	Rod	-	+	-	+	-	-	+	+	-	+			+	
1-5	ERK	+	Rod	-	+	-	+	-	+	+	-	-	+	-		+	*Bacillus licheniformis*
4-2	ERK	+	Rod	-	+	-	+	+	+	-	+	+	+			-	
4-3	ERK	+	Rod	-	+	-	+	+	+	-	+	+	+			-	
8-20	ERK	-	Rod-coccus	-	+	-	+	+	+	+	+	-	+	+	-	+	
8-21	ERK	-	Rod-coccus	-	+	-	+	+	+	+	+	-	+	+	-	+	*Pantoea sp.*
2011					+												
1-2	HL	+	Rod	-	+	-	+	+	+	+	-	-	+				
1-10	HL	+	Rod	+	+	-	-	-	-	+	-	-	-	+		-	*Bacillus sp.*
2-1	HL	+	Rod	-	+	-	+	-	-	-	-	-	-			-	*Arthrobacter phenanthrenivorans*

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Table 4.14. (Cont.)

Isolate name	Source	Gram Stain	Cell Morphogy	Spore	Catalase Activity	Carbohydrate fermentation								VP test	Indole Test	Nitrate test	ID based on DNA sequence analysis
						Lactose	Glucose	Fruktose	Arabinose	Mannitol	Maltose	Cellobiose	Sucrose				
4-24	HL	+	Rod		+	-	-	-	-	+	-	-	-			-	
4-25	HL	+	Rod	+	+	-	-	-	-	-	-	-	-				
4-28	HL	+	Rod	+	+	-	-	-	-	-	-	-	-	+		+	*Bacillus sp.*
5-7	HL	+	Rod	+	+	-	-	-	-	-	-	-	-	+		+	*Bacillus sp.*
6-12	HL	+	Rod	-	+	-	-	-	+	-	-	-	-				
6-13	HL	+	Rod		+	-	+	-	+	+	-	-	-				
6-18	HL	+	Rod		+	-	+	-	-	-	-	-	+				
6-29	HL	+	Rod	-	+	-	-	-	-	-	-	-	-			-	*Arthrobacter sp.*
6-31	HL	-	Rod	-	+	-	+	+	+	+	+	-	+	-		+	*Pantoea sp*
7-4	HL	+	Rod	-	+	-	+	+	+	+	+	-	-				
7-7	HL	-	Rod	-	+	-	-	-	+	-	-	-	-				
7-8	HL	-	Rod	-	+	-	-	-	+	-	-	-	-				
7-9	HL	+	Rod	+	+	-	-	-	-	+	-	-	-	+		+	*Bacillus sp.*
7-10	HL	+	Rod	-	+	-	-	-	+	-	-	-	+				
2012					+												
1-3	HL	-	Rod	-	+	+	+	+	-	+	+	+	+	+	+		
2-4	HL	+	Rod-coccus	-	+	-	-	+	-	-	-	-	-			-	*Curtobacterium sp.*
2-6	HL	-	Rod-coccus	-	+	-	-	+	-	-	-	-	-			-	
2-8	HL	+	Rod	-	+	-	+	+	-	-	-	-	+			-	*Rhodococcus sp.*
3-7	HL	+	Rod	-	+	-	+	+	+	+	-	+	+			+	
3-8	HL	+	Rod	-	+	-	+	w	+	+	-	+	+			-	
3-9	HL	+	Rod	-	+	-	+	+	W	w	-	+	+			+	
3-11	HL	+	Rod	-	+	-	+	+	+	+	-	+	+			+	

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Table 4.14. (Cont.)

Isolate name	Source	Gram Stain	Cell Morphogy	Spore	Catalase Activity	Carbohydrate fermentation								VP test	Indole Test	Nitrate test	ID based on DNA sequence analysis
						Lactose	Glucose	Fruktose	Arabinose	Mannitol	Maltose	Cellobiose	Sucrose				
3-13	HL	-	Rod	-	+	-	+	+	+	+	-	-	+				*Pantoea sp.*
3-14	HL	-	Rod	-	+	-	+	+	+	+	-	-	+				
3-16	HL	-	Rod	-	+	-	+	+	+	+	-	-	+				
3-17	HL	-	Rod	-	+	-	+	+	+	+	-	-	+				
3-18	HL	-	Rod	-	+	-	+	+	+	+	-	-	+				
3-24	HL	+	Rod	+	+	-	+	+	+	-	-	-	-	+	-	+	*Bacillus licheniformis*
4-7	HL	-	Rod	-	+	-	+	-	+	-	-	-	-		-	-	
4-8	HL	+	Rod	-	+	-	+	+	-	-	-	+	+			-	
4-9	HL	+	Rod	-	+	-	+	+	-	-	+	+	+			-	
4-13	HL	-	Rod	-	+	-	+	-	-	-	-	-	-			-	
4-16	HL	-	Rod	-	+	-	+	-	-	-	-	-	-			-	
4-17	HL	-	Rod	-	+	-	+	-	-	-	-	-	-		-	-	
5-8	HL	-	Rod	-	+	-	-	-	-	-	-	-	-		-	-	
5-12	HL	-	Rod	-	+	-	-	-	-	-	-	-	-		-	-	
5-13	HL	-	Rod	-	+	-	+	-	-	-	-	+	-		-	-	
5-19	HL	+	Rod	+	+	-	+	-	+	+	-	-	+	-		+	*Bacillus megaterium*
5-21	HL	+	Rod	+	+	-	+	+	+	+	-	+	+	-		+	
6-1	HL	+	Rod	-	+	-	+	+	-	-	+	+	-			+	
6-2	HL	+	Rod	-	+	-	+	+	-	-	+	-	-			+	
6-3	HL	+	Rod	-	+	-	+	-	-	-	+	+	-			+	
7-10	HL	+	Rod-coccus	-	+	-	-	-	-	-	-	-	-			-	*Bacillus simplex*
7-12	HL	-	Rod	-	+	-	+	-	W	-	-	-	-			+	
7-20	HL	+	Rod	-	+	-	+	-	+	+	W	W	+			-	
8-1	HL	+	Rod	+	+	-	+	+	-	-	-	-	+	+	-	+	Unidentified

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Table 4.14. (Cont.)

Isolate name	Source	Gram Stain	Cell Morphogy	Spore	Catalase Activity	Carbohydrate fermentation								VP test	Indole Test	Nitrate test	ID based on DNA sequence analysis
						Lactose	Glucose	Fruktose	Arabinose	Mannitol	Maltose	Cellobiose	Sucrose				
8-5	HL	+	Rod-coccus	-	+	-	+	+	+	+	+	+	+			-	*Curtobacterium sp.*
8-13	HL	+	Rod	-	+	-	+	-	w	-	-	+	-			-	
8-14	HL	+	Rod	-	+	-	+	+	+	-	+	+	-			+	
2011																	
1A-2	EH	+	Rod	-	+	-	-	-	-	-	-	-	-			-	
1A-3	EH	+	Rod	-	+	-	-	-	-	-	-	-	-			-	*Bacillus simplex*
1A-4	EH	+	Rod	-	+	-	+	+	+	+	-	+	+			-	
1A-9	EH	+	Rod	-	+	-	+	+	+	-	-	-	-			+	
1A-11	EH	+	Rod	+	+	-	+	+	+	+	+	+	+			+	
2A-2	EH	+	Rod	-	+	-	+	+	+	+	+	+	+			+	
2A-3	EH	+	Rod	-	+	-	+	+	+	+	+	+	+			+	
2A-4	EH	+	Rod	-	+	-	-	-	+	+	+	+	+			+	*Arthrobacter sp*
4A-3	EH	-	Rod	-	+	-	-	-	+	+	+	+	+		-	+	
5A-7	EH	+	Rod	-	+	-	-	-	-	-	-	-	-				
5A-9	EH	+	Rod	+	+	-	+	+	+	-	-	-	-	+	-	+	*Bacillus licheniformis*
5A-10	EH	-	Rod	-	+	-	+	-	+	+	+	+	-		-	-	
6A-7	EH	+	Rod	-	+	-	+	-	-	-	-	-	-			+	*Arthrobacter sp.*
7A-3	EH	+	Rod	-	+	-	-	+	+	-	-	-	+			-	
7A-4	EH	+	Rod	-	+	-	-	-	-	-	-	-	+			+	
2012																	
1A-1	EH	-	Rod	-	+	-	-	-	-	-	-	-	-			-	
1A-4	EH	-	Rod	-	+	-	+	-	-	-	-	-	-			+	
1A-5	EH	+	Rod	-	+	-	+	-	+	+	-	-	+			-	

(Cont. on next page)

Table 4.14. (Cont.)

Isolate name	Source	Gram Stain	Cell Morphogy	Spore	Catalase Activity	Carbohydrate fermentation								VP test	Indole Test	Nitrate test	ID based on DNA sequence analysis
						Lactose	Glucose	Fruktose	Arabinose	Mannitol	Maltose	Cellobiose	Sucrose				
1A-6	EH	+	Rod	+	+	+	+	+	+	+	-	-	+	-		+	
1A-8	EH	+	Rod	+	+	-	+	-	+	+	-	-	+	-			
1A-11	EH	+	Rod	+	+	-	+	+	+	-	-	-	+	+	-	+	
2A-6	EH	+	Rod	+	+	-	+	+	+	+	-	+	-	+	-	+	*Bacillus subtilis*
2A-7	EH	-	Rod	-	+	+	+	+	+	-	+	w	-		+	+	
2A-23	EH	+	Rod	+	+	+	+	-	+	-	+	+	+	-		+	*Bacillus litoralis*
3A-10	EH	+	Rod	+	+	+	+	-	+	+	+	-	-	-	-	+	*Bacillus megaterium*
3A-11	EH	+	Rod	-	+	+	+	+	+	-	+	w	-		+	+	
3A-15	EH	+	Rod	+	+	-	+	-	+	+	-	-	+	-		+	*Bacillus sp.*
3A-21	EH	+	Rod	+	+	-	+	+	-	+	-	-	+	-			
4A-1	EH	+	Rod	+	+	-	+	+	+	-	-	-	+	+	-	+	*Bacillus cereus*
4A-9	EH	+	Rod-coccus	-	+	-	+	+	-	+	-	-	+			+	*Arthrobacter humicola*
4A-20	EH	+	Rod	-	+	-	+	+	-	+	-	-	+				
5A-2	EH	+	Rod		+	-	+	+	-	+	-	-	+				
5A-10	EH	+	Rod	-	+	-	+	-	+	+	-	-	-			+	
6A-3	EH	+	Rod	-	+	-	+	-	+	+	-	-	+			+	*Arthrobacter phenanthrenivorans*
7A-1	EH	+	Rod-coccus	-	+	-	+	+	-	+	-	-	+			+	*Arthrobacter humicola*
8A-3	EH	+	Rod		+	-	+	-	-	-	+	-	+			+	
8A-11	EH	+	Rod	-	+	-	+	-	-	-	-	-	-		-		
8A-13	EH	+	Rod	-	+	-	+	+	+	-	+	+	+	-	-	-	*Microbacterium hydrocarbonoxydans*
8A-16	EH	+	Rod	-	-	-	+	+	-	-	+	+	-	-		-	

Table 4.15. Physiological and Biochemical Tests Results of Coccus Shaped Bacteria

Isolate name	Source	Gram Stain	Cell Morphogy	Spore	Catalase Activity	Carbohydrate fermentation								Pigment	Indole Test	Nitrate test	ID based on DNA sequence analysis
						Lactose	Glucose	Fruktose	Arabinose	Mannitol	Maltose	Cellobiose	Sucrose				
2011																	
2-15	HO	-	Coccus	-	+	-	-	-	-	-	-	-	-	-		-	
3-9	HO	-	Coccus	-	+	-	-	-	-	-	-	-	-	-		-	
3-10	HO	-	coccus	-	+	-	-	-	-	-	-	-	-	-		-	
5-4	HO	-	Coccus	-	+	-	+	-	+	-	-	-	-	-		-	*Acinetobacter sp.*
5-11	HO	-	Coccus	-	+	-	-	-	-	-	-	-	-	-		-	
5-14	HO	-	coccus	-	+	-	-	-	-	-	-	-	-	-		-	
8-15	HO	-	Coccus	-	+	-	-	-	-	-	-	-	-	-		-	
8-22	HO	-	coccus	-	+	-	+	+	+	+	+	-	-	-		+	
2-41	ERK	-	Coccus	-	+	-	-	-	-	-	-	-	-	-		-	
2-49	ERK	-	Coccus	-	+	-	+	-	+	-	-	-	+	-		-	
3-2	ERK	+	Coccus	-	+	-	-	-	-	-	-	-	-	-		-	*M. luteus*
2012																	
1-7	ERK	+	Tetrad	-	+	-	-	-	-	-	-	-	-	+			*M. luteus*
1-8	ERK	+	Tetrad	-	+	-	-	-	-	-	-	-	-	+			
2011																	
1-11	HL		Coccus	-	+	-	-	-	-	-	-	-	-	-			
2-36	HL		Coccus	-	+	-	-	-	-	-	-	-	-	-			
2-39	HL		Coccus	-	+	-	-	-	-	-	-	-	-	-			
3-17	HL	+	Tetrad	-	+	-	+	+	-	-	-	-	-	+			*M. luteus*
3-18	HL	+	Tetrad	-	+	-	+	+	-	-	-	-	-	+			

(Cont. on next page)

Table 4.15. (Cont.)

Isolate name	Source	Gram Stain	Cell Morphogy	Spore	Catalase Activity	Carbohydrate fermentation								Pigment	Indole Test	Nitrate test	ID based on DNA sequence analysis
						Lactose	Glucose	Fruktose	Arabinose	Mannitol	Maltose	Cellobiose	Sucrose				
4-17	HL	+	Tetrad	-	+	-	+	+	-	-	-	-	-	+			
4-18	HL	+	Tetrad	-	+	-	+	+	-	-	-	-	-	+			
4-20	HL	+	Tetrad	-	+	-	+	+	-	-	-	-	-	+			
7-2	HL	+	Coccus	-	+	-	+	+	+	+	-	-	-	+		+	*Streptomyces sp.*
2012																	
8-12	HL	+	Tetrad	-	+	-	+	+	+	-	-	+	+	-		-	*Staphylococcussp.*
2011																	
5A-2	EH	-	Coccus	-	+	-	-	-	-	-	-	-	-	-	-	-	
7A-8	EH	-	Coccus	-	+	-	-	-	-	-	-	-	-	-	-	-	

Table 4.16. Physiological and Biochemical Tests Results of Bacteria Belongs to *Enterobacteriaceae* Family

Isolate name	Source	Gram Stain	Cell Morphogy	Catalase Activity	Carbohydrate fermentation								VP test	Indole Test	Citrate test	Urea test	H2S Production	Motility test	Related Genus/Species
					Lactose	Glucose	Fruktose	Arabinose	Mannitol	Maltose	Cellobiose	Sucrose							
2011																			
4V-5	HO	-	Rod	+	-	+	+	+	+	+	+	+		-	-	-	-	+	
4V-6	HO	-	Rod	+	-	+	+	+	+	+	+	+		-	-	-	-	+	
4V-9	HO	-	Rod	+	-	+	+	+	+	+	+	+		-	-	-	-	+	
5V-5	HO	-	Rod	+	-	+	+	+	-	+	-	+		-	-	-	-	-	
5V-6	HO	-	Rod	+	-	+	+	+	+	+	-	+		-	-	-	-	-	
5V-7	HO	-	Rod	+	-	+	+	-	+	+	-	+		-	-	-	-	-	
8V-3	HO	-	Rod	+	-	+	+	+	+	+	+	+		-	-	-	-	-	
8V-5	HO	-	Rod	+	-	+	+	+	+	+	+	+		-	-	-	-	-	
8V-7	HO	-	Rod	+	-	+	+	+	+	+	+	+		-	-	-	-	+	
2012																			
5V-1	HO	-	Rod	+	-	+	+	+	+	+	-	+		-	+	-	-	+	
8V-1	HO	-	Rod	+	-	+	+	+	-	+	-	+		-	-	-	-	+	
8V-2	HO	-	Rod	+	-	+	+	+	W	+	-	+		-	-	-	-	-	
2011																			
2V-1	ERK	-	Rod	+	-	+	+	+	+	-	+	+	-	-	-	-	-	+	
2V-2	ERK	-	Rod	+	+	+	+	+	+	+	+	+	+	+	+	-			*K. oxytoca*
2V-3	ERK	-	Rod	+	-	+	+	+	+	-	+	+	-	-	-	-	-	+	
2V-4	ERK	-	Rod	+	+	+	+	+	+	+	+	+	+	+	+	-			
2V-5	ERK	-	Rod	+	-	+	+	+	+	-	+	+	-	-	-	-	-	+	
4V-2	ERK	-	Rod	+	-	+	+	+	+	-	+	+	-	-	-	-	-	+	
2011																			
7V-7	HL	-	Rod	+	-	+	+	+	+	-	-	+	-	-	-	-	-	+	

(Cont. on next page)

Table 4.16. (Cont.)

Isolate name	Source	Gram Stain	Cell Morphogy	Catalase Activity	Carbohydrate fermentation								VP test	Indole Test	Citrate test	Urea test	H2S Production	Motility test	Related Genus/Species
					Lactose	Glucose	Fruktose	Arabinose	Mannitol	Maltose	Cellobiose	Sucrose							
7V-9	HL	-	Rod	+	-	+	+	+	+	-	-	+	-	-	-	-	-	-	
2012																			
2V-1	HL	-	Rod	+	-	+	+	+	+	-	-	+	-	-	-	-	-	+	
2V-2	HL	-	Rod	+	-	+	+	+	+	+	-	+	-	-	-	-	-	+	
2011																			
A1V-3	EH	-	Rod	+	-	+	+	-	-	+	-	+	-	-	-	-	-	-	
A1V-5	EH	-	Rod	+	-	+	+	+	+	+	-	+	-	-	-	-	-	-	
A5V-2	EH	-	Rod	+	-	+	+	+	+	+	-	+	-	-	-	-	-	+	
A6V-7	EH	-	Rod	+	-	+	+	+	+	+	-	+	-	-	-	-	-	-	
A7V-2	EH	-	Rod	+	-	+	+	+	+	+	-	+	-	-	-	-	-	+	
A7V-8	EH	-	Rod	+	-	+	+	+	+	+	-	+	-	-	-	-	-	+	

4.6. Molecular Identification of Bacteria

4.6.1. Isolation of Genomic DNA

Genomic DNA of samples were isolated as described by Cardinal et al. (1997). Isolation of genomic DNA was controlled by a spectrophotometer (Nano Drop 8000, Thermo Scientific, Wilmington, USA).

4.6.2. Amplification of 16S rDNA Region

25 µl of PCR mixture including 5µl of genomic DNA was amplified by PCR. Two different PCR mixture was prepared; one of them was including primers of EGE1-EGE2 and the other was including 341F-518R primers. Amplification by both primers were succesfully performed but in further analysis only 341F-518R were evaluated. Amplification of genomic DNA was visualized by agarose gel electrophoresis under UV light. The length of base pairs of amplified PCR products were observed between 100 bp and 200 bp. The following images of agarose jel electrophoresis belong to amplified PCR product by 341F-518R primers (Figure 4.3, Figure 4.4 and Figure 4.5). *Escherichia coli* was used as a reference strain.

25µL genomic DNA samples were amplified by PCR and amplification of genomic DNA was visualised by agarose gel electrophoresis under UV light. Gel electrophoresis were performed with three groups of randomly chosen samples. The length of base pairs of amplified PCR products was observed between 100 bp and 200 bp. The following images of agarose gel electrophoresis belong to amplified PCR product by 341F-518R primers (Figure 4.3, Figure 4.4 and Figure 4.5).

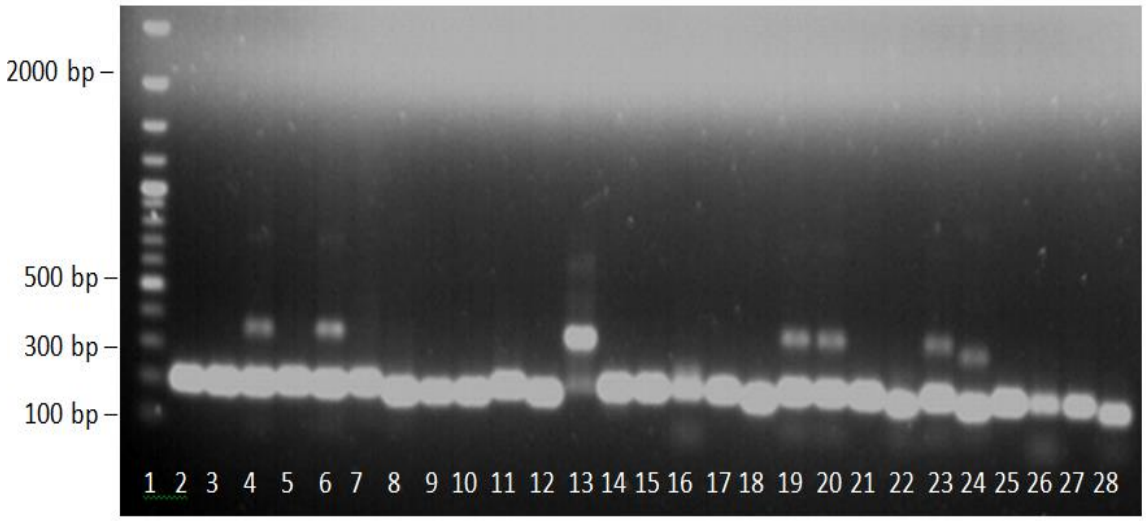

Figure 4.3. First sample group's amplified products of by 518R-341R primers; **Lanes: 1.** 100 bp DNA ladder**, 2.** 1-10 (*Bacillus simplex*); **3.** 1-26 (*Bacillus simplex*); **4.** A1V-5**; 5.** 2-19 (*Klebsiella oxytoca*); **6.** 2-35 (*Pantoea agglomerans*); **7.** 2V-2 (*Klebsiella oxytoca*); **8.** 2A-4 (*Arthrobacter sp.)*; **9.** 3-2 (*Micrococcus luteus*); **10.** 3-17 (*Micrococcus luteus*); **11.** 4-28 (*Bacillus sp.*,); **12.** 4-18; **13.** 5-4 (*Acinetobacter sp.*); **14.** 5A-2; **15.** 5A-9 (*Bacillus licheniformis*); **16.** 5A-10; **17.** 5A-11; **18.** 6-29 (*Arthrobacter sp.*); **19.** 6-25; **20.** 6-24; **21.** 6-18; **22.** 6A-7 (*Arthrobacter sp*.); **23.** 6-31 (*Pantoea sp.*); **24.** 7-2 (*Streptomyces sp*.); **25.** 7-13(*Baciluus sp.*); **26.** 8A-7; **27.** 8-20 (*Pseudomonas sp.*); **28.** 7A-4

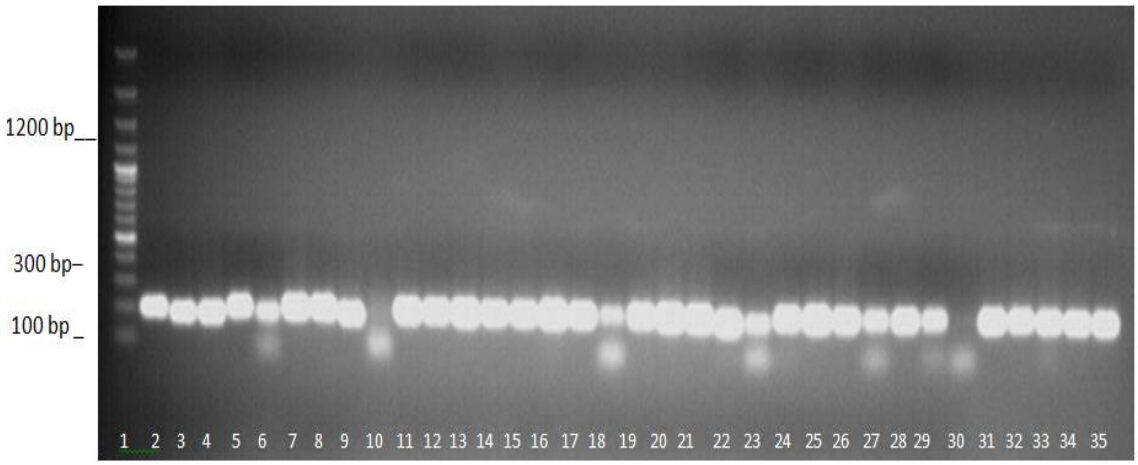

Figure 4.4. Second sample group's amplified products by 518R-341R primers; **Lanes**: **1.** 100 bp DNA ladder, **2.**1A-9; **3.** 1-7 (*Micrococcus sp.*,); **4.** 1-8; **5.** 2A-6 (*Bacillus subtilis*); **6.** 2A-20; **7.** 2A-21; **8.** 2A-23 (*Bacillus litoralis*); **9.** 2-4 (*Curtobacterium sp.*); **10.** 2-8 (*Rhodococcus sp.*) **11.** 3-8; **12.** 3A-10 (*Bacillus megaterium)* ; **13.** 3A-13; **14.** 3A-15 (*Bacillus sp.*); **15.**3-24 (*Bacillus licheniformis*); **16.** 3A-7; **17.** 3-23 (*Bacillus subtilis*); **18.** 4-8; **19.** 4A-1 (*Bacillus cereus*); **20.** 5-4; **21.** 5A-14; **22.** 5-8; **23.** 5-13; **24.** 5-1 (*Pantoea sp.*); **25.** 6-3; **26.** 6-15 (*Klebsiella pneumoniae*); **27.** 6A-9; **28.** 7-10 (*Bacillus simplex*); **29.** 8-1(Not determined); **30.** 8-13; **31.** 8-12 (*Staphylococcus sp.*); **32.** 8-21 (*Pantoea sp.*); **33.** 8A-16; **34.** 6A-4; **35.** *E.coli*

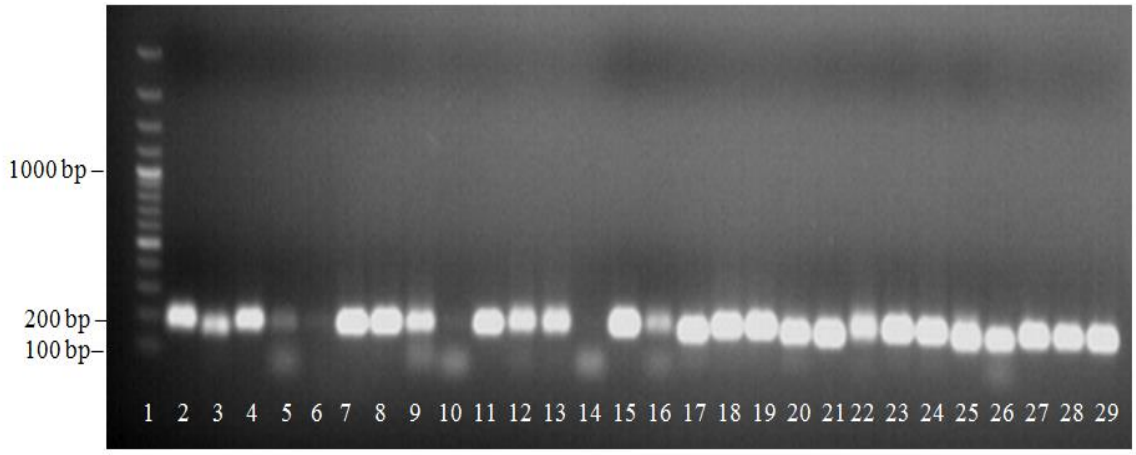

Figure 4.5. Third samples group's amplified products by 518R-341R primers; **Lanes: 1.** 100 bp DNA ladder, **2.** 1A-3 (*Bacillus simplex*); **3.** 2-1(*Rhodococcus sp.*); **4.** 2-15; **5.** 2-49; **6.** 4-9; **7.** 5-7 (*Bacillus sp.*); **8.** 5-13 (*Pantoea agglomerans*); **9.** 4-14 (*Pantoea sp.*); **10.** 6-7; **11.** 7-9 (*Bacillus sp.);* **12.** 8-16; **13.** 8-17 (*Pseudomonas sp.*); **14.** 8-28; **15.** 3-13; **16.** 4-7; **17.** 4A-9 (*Arthrobacter humicola*) ; **18.** 5-19 (*Bacillus megaterium*); **19.** 7-2 (*Pantoea agglomerans*); **20.** 7A-1 (*Arthrobacter humicola*); **21.** 8-5 (*Curtobacterium sp.*); **22.** 8-10; **23.** 8-24 (*Pantoea agglomerans*); **24.** 8-25 (*Pantoea agglomerans*); **25.** 8A-13 (*Microbacterium hydrocarbonoxydans*); **26.** 6A-3 (*Arthrobacter phenanthrenivorans*); **27.** 5A-12; **28.** 1-5 (*Bacillus licheniformis*); **29.** *E.coli*

4.6.3. Identification of Bacteria by DNA Sequencing Methods

As mentioned before, amplifying the genomic DNA was successfully performed by both primers, 341F- 518R and EGE1-EGE2 primers. Since the sequencing is performed in only one direction, 341F primer was preferred coincidently. The representative strains were chosen randomly according to their frequency of occurrence and also rarely seen isolates were all subjected to DNA sequencing analysis.

4.6.3.1. Results for Hurma Olive

Strains originated from Hurma olive were subjected to DNA sequence. In both seasons, almost the same bacteria were identified with high scores (93-100 %) that was shown in Table 4.17. Especially, *Pantoea agglomerans* is mostly isolated from plant surfaces (Winn and Koneman, 2006) and had the highest identification score (100%). It was the most isolated bacteria in both season and followed by *Bacillus sp.* In addition, *Klebsiella sp*. as a member of lactose positive *Enterobacteriaceae,* was isolated in both seasons. Even if no *Pseudomonas sp.* growth occurred on Pseudomonas agar, DNA sequenced-identified Gram-negative bacteria belong to genus of *Pseudomonas;* also another identified Gram-negative bacterium was *Acinetobacter sp*. which were isolated only in Hurma olive in 2011. Our results are almost different from the findings of Fakas et al. (2010) for Greek olive fruit of Amfissis variety which was characterized in respect of bacterial microflora from two batches: at the beginning (A) and at the end (B) of the harvest. In this study, batch B has same ripeness level as Hurma olives. In that bacth (B) olives' microflora included *Caseobacter spp., Cellulomonas sp., Corynebacterium spp., Alcaligenes spp., Franciscella spp., Vibrio spp., Xanthomonas spp.* and dominantly *Pseudomonas spp.* But in batch A, *Acinetobacter spp*. and *Pseudomonas spp*. were identified similar to our results. In another study of Mantzouridou and Tsimidou (2011), lyophilized Thassos variety of olive fruits at the stage of over ripeness were investigated prior to processing of hot air drying. Differently from our findings, in this study, raw olive fruits' microflora comprised of mainly lactic acid bacteria and yeasts, but *Enterobacteriaceae, S. aureus, Bacillus spp*. and *Clostridium spp.* were undetectable. Overall, Hurma olive had a difference in addition to similarities in respect of microflora.

These differences in composition of olive microflora could be arisen from the olive fruit variety.

Although pseudomonads did not occur in related agar media during maturation period, they were identified at the end of the DNA sequencing analysis. This was due to low level of pseudomonads which remained below the detection limit.

Table 4.17. Identified bacteria originated from Hurma olive by DNA sequencing method

Code	Biochemical ID[a]	Genus/Species	MAX ID (%)	GenBank[b]
		2011		
1-26	*Bacillus cereus*	*Bacillus simplex*	100	KC936232
2-19	*Klebsiella oxytoca*	*Klebsiella oxytoca*	98	KC593550
4-14	*Unidentified*	*Pantoea sp.*	100	KC252899
5-4	*Unidentified*	*Acinetobacter sp.*	93	HQ449642
5-13	*Unidentified*	*Pantoea agglomerans*	100	KC283056
7-13	*Bacillus sp.*	*Bacillus sp.*	99	HM804387
8-17	*Unidentified*	*Pseudomonas sp.*	97	DQ357702
8-20	*Neisseria sp.*	*Pseudomonas sp.*	100	HQ403168
		2012		
3-23	*Bacillus subtilis*	*Bacillus subtilis*	99	GQ392055
5-1	*Unidentified*	*Pantoea sp.*	100	JN853224
6-15	*Klebsiella pneumoniea subsp. ozaenae*	*Klebsiella pneumoniea*	100	JX069939
7-2	*Unidentified*	*Pantoea agglomerans*	100	KC936207
8-24	*Unidentified*	*Pantoea agglomerans*	100	KC936207
8-25	*Unidentified*	*Pantoea agglomerans*	100	KC936207

[a] Identified according to biochemical test results
[b] Accession number of 16s rRNA sequences in GenBank

4.6.3.2 Results for Non-Debittered Erkence Olive

Since microbial load of Erkence olive was at the low level compared to Hurma olive, their isolated culture amounts were also lower than Hurma olives'. When the Table 4.18 is evaluated, except for *Pantoea spp.*, the rest of identified bacteria samples were in accordance with their results of biochemical identification at the level of genus. In both seasons, biochemically identified *Micrococcus luteus* were present in the microflora of non-debittered Erkence olive and this result also was confirmed by DNA sequencing. These yellow pigmented Gram-positive tetrad formed cocci are predominantly found in mammalian skin and in soil, but commonly isolated from food product and the air (Buchanan and Gibbons, 1974). In 2011 members of

Enterobacteriaceae, *Pantoea spp.* and *Klebsiella oxytoca* were identified by DNA sequencing method. In addition, the genus of bacteria identified in both seasons was *Pantoea sp*. The biochemical tests were insufficient to identify *Pantoea spp.*. Moreover *Bacillus licheniformis* is saprophytic bacterium and widely distributed in environment (Rey et. al., 2004) which was also present in non-debittered Erkence olive microflora. Fakas et al. (2010) determined the microflora of Amfissis olive variety at the beginning of maturation including *Actinomyces spp, Corynebacterium spp. Rhodococcus spp., Streptococcus spp, Listeria spp., Microbacterium spp., Acinetobacter spp., Pseudomonas spp. and Achromobacter spp.*. In another study about the microflora of green olives of Bella di Cerignola variety's characterization of bacterial microflora *Bacillus spp.* were presented as a dominant group; as the fermentation continued *Enterobacter cloacae, Enterobacter amnigenus, Chryseobacterium spp*. and *Bacillus subtilis* were isolated and identified (Campaniello et al., 2005). This output about *Bacillus spp*. explains why genus of *Bacillus* was mainly isolated and identified from all kinds of samples used in our study.

Table 4.18. Identified bacteria originated from non-debittered Erkence olive by DNA sequencing method

Code	Biochemical ID[a]	Genus/Species	MAX ID (%)	GenBank[b]
		2011		
2-35	*Enterobacter intermedius*	*Pantoea agglomerans*	99	KC178591
2V-2	*Klebsiella oxytoca*	*Klebsiella oxytoca*	98	JX267073
3-2	*Micrococcus luteus*	*Micrococcus luteus*	100	HF952653
		2012		
1-5	*Bacillus sp.*	*Bacillus licheniformis*	99	HQ858064
1-7	*Micrococcus luteus*	*Micrococcus sp.*	100	DQ659067
8-21	*Unidentified*	*Pantoea sp.*	100	KC252899

[a] Identified according to biochemical test results
[b] Accession number of 16s rRNA sequences in GenBank

4.6.3.3. Results for Leaf of Erkence Cultivar

Leaves constitute various large bacterial habitats. Bacteria are main inhabitant of phylloplane and their number could reach to 10^7 cells/m^2 of leaf (Lindow and Brandl, 2002). More diversified genera of bacteria were isolated from leaf samples during maturation (Table 4.19). The common bacteria identified in both seasons belonged to genera of *Bacillus*, *Pantoea* and *Rhodococcus.* Apart from the previously mentioned

samples, in the microflora of leaves, Gram-positive rod-coccus shaped and Gram-positive coccus shaped bacteria were identified at the level of genera *Arthrobacter sp.*, *and Streptomyces sp.*, respectively. *Arthrobacter* is abundant in environment, especially in soils (Buchanan and Gibbons, 1974). Besides, *M. luteus* was identified in leaf samples alike in non-debittered Erkence olive.

In 2012, completely different genera were also identified from previously mentioned genera: *Rhodococcus, Curtobacterium, Staphylococcus*. The first one is in coryneform genera. *Rhodococcus sp*. is distributed widely in soil where they degrade a wide range of organic compounds and also a diversified substrate range is exhibited by rhodococci for degradation of phenols, aromatic acids, halogenated phenols etc. (Collins,2006; McLeod et al,2006; Finnerty, 1992). Another isolated genus *Staphylococcus sp.* is mainly found in skin and mucous membranes of warm-blooded animals but are generally isolated from food, dust and water (Buchanan and Gibbons, 1974). Although staphylococci and micrococci did not grow on Baird-Parker agar plates and remained below the detection limit; they were identified by DNA sequencing method. On the other hand, some species of *Curtobacterium* are associated with plants. Some of them are pathogen and cause disease invading plant tissues while some of them saprophyte that not cause a disease but use leaf and root surfaces (Dunleavy, 1989). Moreover the common bacteria were found in leaf samples such as *B. licheniformis*, *B. simplex*, *B.megaterium* and genus of *Pantoea sp.* which are previously identified in the samples of non-debittered Erkence olives and Hurma olives.

Unique study about olive leaf microflora of Ercolani (1991) is in conformity with our findings. In that study, during six seasons, frequency of bacteria of phylloplane was investigated. Therefore, *Bacillus megaterium, Micrococcus luteus, Curtobacterium sp., Arthrobacter sp. and Bacillus subtilis* are the common genera and species found among identified bacteria in leaves of Erkence olive variety and Ercolani's study. Besides, *staphylococci, pseudomonads, xanthomonads, listeriae and pink chromogens* bacteria are listed as mostly growing microorganisms on the plant surfaces; the latter is protected by the formation of pigment against the exposure of sun light (Mohapatra, 2008). Moreover difference of communities inhabiting on leaf surfaces depend on various factor such as age of leaf, variety of olive, meteorological conditions. However the most of the phyllosphere microbes found in nature are allochtonous and are transferred from soil, water, air to their phyllosphere habitat or vice verca (Mohapatra, 2008). Phyllosphere is mostly associated with soil microbes since they are deposited on

this habitat by dispersing from soil (Mohapatra, 2008). Similar to our results, Yoshimura (1982) found *Bacillus sp.* in both leaves of *Pinus densiflora* and in the air of the pine forest all year round while coryneform bacteria were observed throughout the year in the air of the same pine forest.

Table 4.19. Identified bacteria originated from leaves of Erkence olive variety by the method of DNA sequencing

Code	Biochemical ID[a]	Genus/Species	MAX ID (%)	GenBank[b]
		2011		
1-10	*Bacillus sp.*	*Bacilllus simplex*	98	KC936232
2-1	*Unidentified*	*Rhodococcus sp.*	95	DQ272471
3-17	*Micrococcus luteus*	*Micrococcus luteus*	98	HF952653
4-28	*Bacillus cereus.*	*Bacillus sp.*	100	AM910170
5-7	*Bacillus sp.*	*Bacillus sp.*	88	KC540840
6-29	*Unidentified*	*Arthrobacter sp.*	100	KC522130
6-31	*Unidentified*	*Pantoea sp.*	99	JX908928
7-2	*Micrococcus varians*	*Streptomyces sp.*	100	JF903932
7-9	*Bacillus sp.*	*Bacillus sp.*	99	AM910170
		2012		
2-4	*Unidentified*	*Curtobacterium sp.*	100	KC466122
2-8	*Unidentified*	*Rhodococcus sp.*	84	DQ272471
3-13	*Unidentified*	*Pantoea sp.*	100	KC311262
3-24	*Bacillus sp.*	*Bacillus licheniformis*	100	HQ858064
5-19	*Bacillus megaterium*	*Bacillus megaterium*	100	JX274543
7-10	*Unidentified*	*Bacillus simplex*	99	KC692191
8-1	*Bacillus cereus*	*Unidentified*		
8-5	*Unidentified*	*Curtobacterium sp.*	90	KC466121
8-12	*Staphylococcus sp.*	*Staphylococcus sp.*	100	KC492525

[a] Identified according to biochemical test results
[b] Accession number of 16s rRNA sequences in GenBank

4.6.3.4 Results for Air Samples from Erkence Olive Orchard

Identified bacteria from sedimented air samples were given in Table 4.20. Noticeably all identified bacteria by DNA sequencing are Gram-positive. In 2011, *Bacillus licheniformis*, *Bacillus simplex* and *Arthrobacter sp.* were listed as a result of identification. These organisms are indigenous in environment especially in soil (Fakas et al., 2010; Rey et al., 2004; Buchanan and Gibbons, 1974) and may have been transferred via air from the soil.

In 2012, more isolates were subjected to DNA sequencing and concluded that identified genera Gram positive rod-coccus and bacilli were predominant in the microflora of Erkence olive orchard's air: *Arthrobacter sp.* and *Bacillus sp.* The species of *B. cereus, B. litoralis, B.megaterium and B.subtilis* were identified in addition to them, soil indigenous bacteria *Arthrobacter humicola*, and phenanthrene (PAH derivatives, as pollutants, is found mainly in soil contaminated by industrial activities) degrading *A. phenanthrenivorans* (Vandera et al,2012) and crude-oil degrading *Microbacterium hydrocarbonoxydans* (Schippers et al, 2005) were identified from air samples. Comparison to a microbiological characterization study of olive orchard is not possible since no literature was available. But a similar study was performed with plum orchard air samples (Tuszynski and Satora, 2003). In that study *Bacillus sp., Micrococcus sp.* and *Staphylococcus sp.* were dominated in the microflora of plum orchard while *Bacillus sp*. was determined as the most dominated genus in the microflora of orchard of Erkence variety olive. *Bacillus sp.* was also occurred in the air samples of pine forest (Yoshimura, 1982). Since, the air microflora are composed of soil and phyllosphere microflora, it is not suprising to find similar bacteria in air microflora and leaf microflora. Overall, air is not so effective on formation of Hurma olive since it is a transmission zone between soil and phyllosphere. Main habitat of identified bacteria is determined as soil. In addition, leaf and olive fruits are present in the same environment that explains similar bacterial microflora found in all samples.

Table 4.20. Identified bacteria originated from air samples from of Erkence olive orchard by the method of DNA sequencing

Code	Biochemical ID[a]	Genus/Species	MAX ID (%)	GenBank[b]
		2011		
1A-3	*Unidentified*	*Bacillus simplex*	99	KC69219
2A-4	*Unidentified*	*Arthrobacter sp.*	100	HQ333015
5A-9	*Bacillus sp.*	*Bacillus licheniformis*	100	GQ169102
6A-7	*Unidentified*	*Arthrobacter sp.*	99	KC522130
		2012		
2A-6	*Bacillus subtilis*	*Bacillus subtilis*	100	GQ392049
2A-23	*Bacillus sp.*	*Bacillus litoralis*	95	JF411239
3A-10	*Bacillus megaterium*	*Bacillus megaterium*	99	JX274543
3A-15	*Bacillus megaterium*	*Bacillus sp.*	99	HM804387
4A-1	*Bacillus cereus*	*Bacillus cereus*	100	KC540842
4A-9	*Unidentified*	*Arthrobacter humicola*	96	JX401463
6A-3	*Unidentified*	*Arthrobacter phenanthrenivorans*	100	KC934897
7A-1	*Unidentified*	*Arthrobacter humicola*	97	JX401463
8A-13	*Unidentified*	*Microbacterium hydrocarbonoxydans*	100	KC934825

[a] Identified according to biochemical test results

[b] Accession number of 16s rRNA sequences in GenBank

4.7. Sequencing of Isolates

Phylogenetic trees were conducted using the neighbor-joining method and analyzed with MEGA 5 software (Tamura et al., 2011). 16S rDNA sequence analysis showed that the strains of Hurma olive in both years have the highest similarity with the values of 98% and 95% (Figure 4.6). 16 S rDNA gene sequence similarity of the isolated *Bacillus* and *Pantoea* type strains were the highest in Hurma olive (98%) (Figure 4.6, A) and *Micrococcus* type strains showed 95% homology between each other (Figure 4.6, B).

16 S rDNA gene sequence similarities of the isolated bacteria from the leaves of Erkence cultivars (A) and air of Erkence olive orchard (B) were investigated by phylogenetic trees (Figure 4.7). According to sequencing results of *Arthrobacter*, *Curtobacterim, Pantoea* and *Bacillus* type strains showed 98% homology (Figure 4.7.A). Besides, each group of *Bacillus* and *Arthrobacter* shared high degree of sequence similaritiy (95%) of 16S r DNA (Figure 4.7. B).

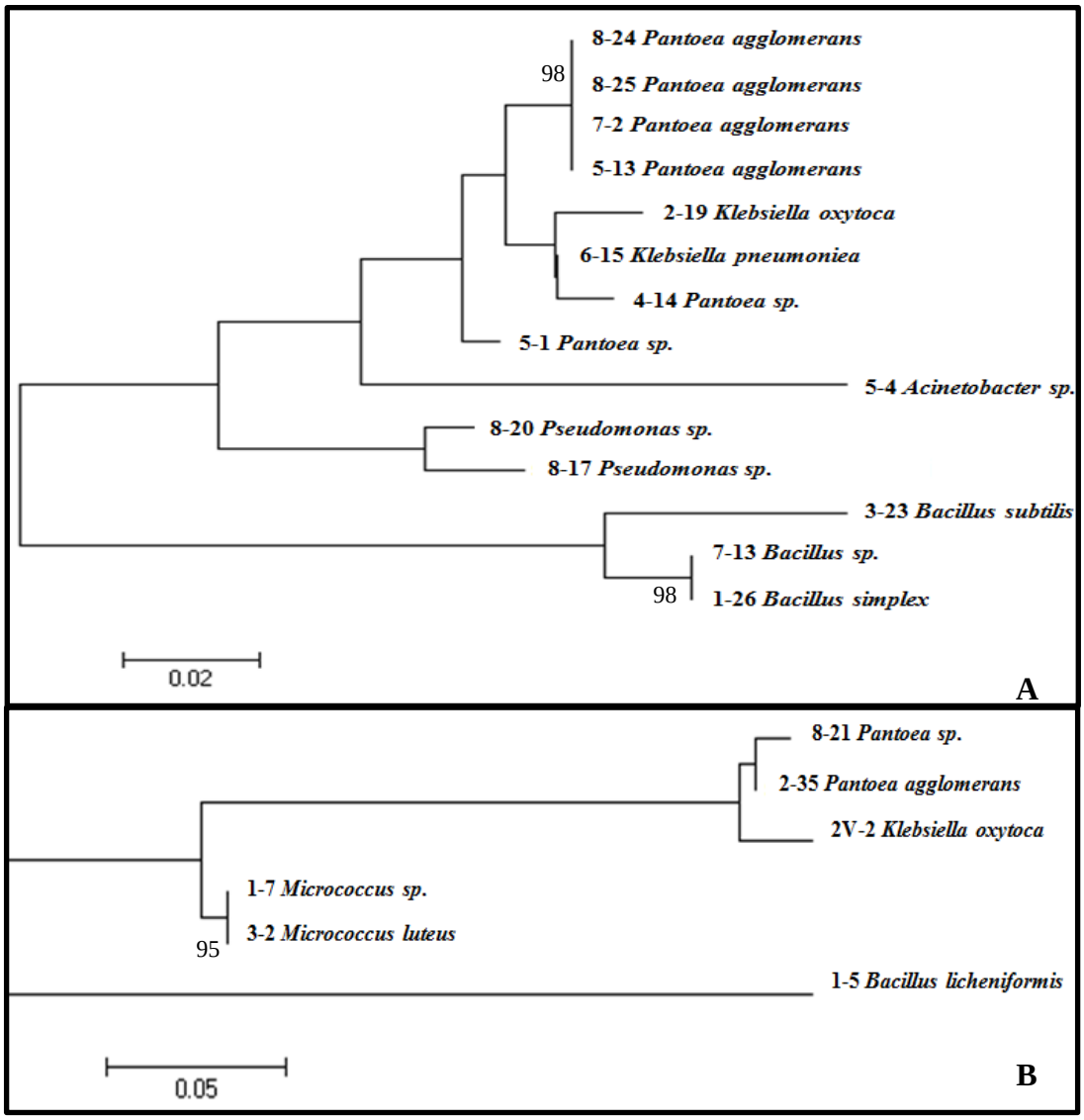

Figure 4.6. Phylogenetic (neighbor-joining) trees showing the relationships between sequenced bacteria isolated from Hurma olive (A) and non-debittered Erkence olive (**B**) during maturation period (between the start of debittering to full ripeness) in both crop years, based on the 16S r DNA gene sequences. The scale bars represent 2 % (A) and 5% (B) divergence.

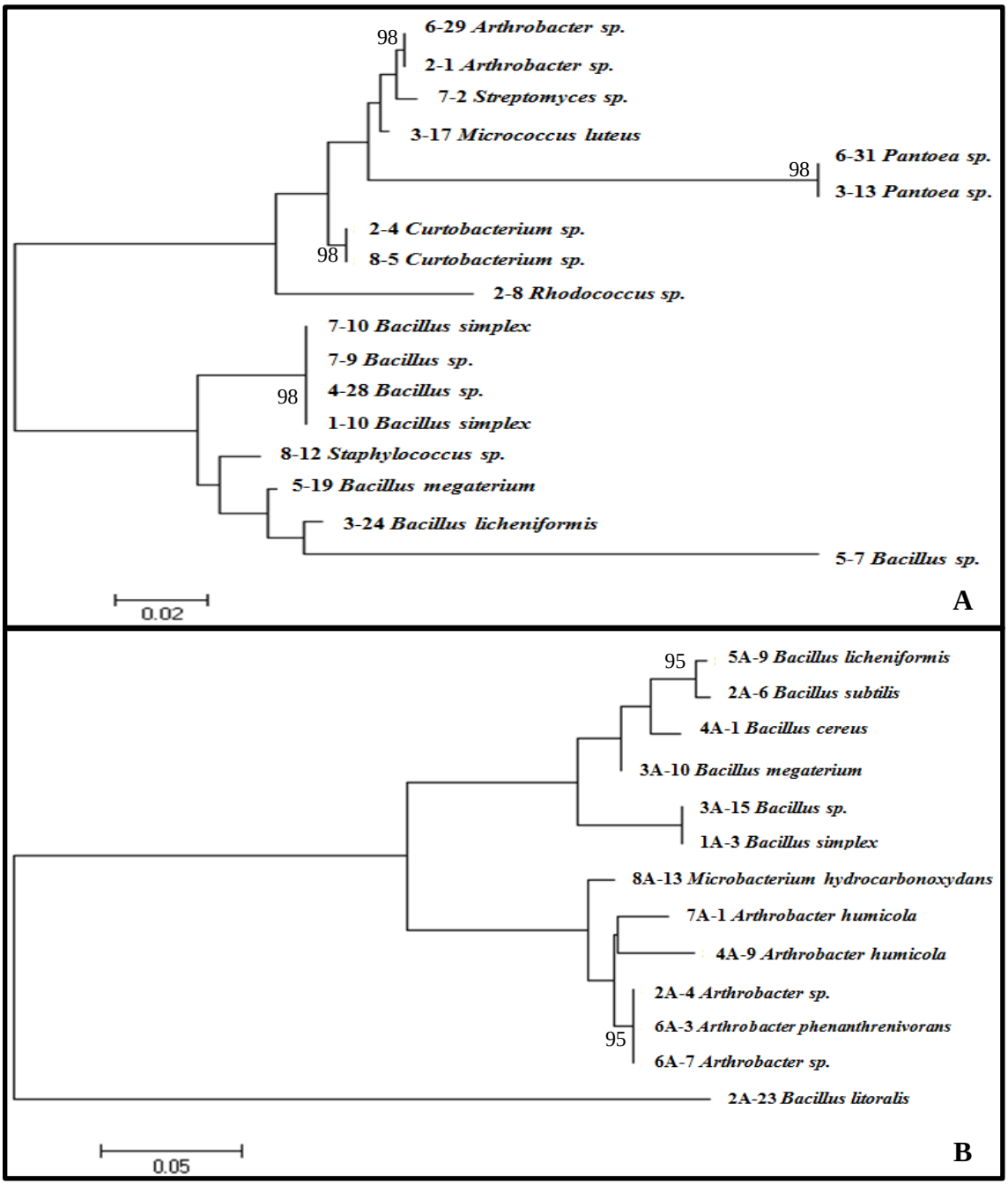

Figure 4.7. Phylogenetic (neighbor-joining) trees showing the relationships between sequenced bacteria isolated from leaves of Erkence cultivar (A) and air of Erkence olive orchard (B) during maturation period (between the start of debittering to full ripeness) in both crop years, based on the 16S rDNA gene sequences. The scale bars represent 2 % (A) and 5% (B) divergence.

CHAPTER 5

CONCLUSION

Bacterial characterization of Hurma olive, non-debittered Erkece olive, leaf and orchards' air microflora and microbial population during its maturation period were the main aim of this study. For this aim, firstly maturation period was monitored and population dynamic of Hurma olive was compared to Gemlik olive's in terms of bacterial and fungal growth. After monitoring growth during maturation, bacteria were isolated from counting media namely PCA and VRBGA. In the first season compared to second season, aerobic mesophilic, yeast-mold and *Enterobacteriacaae* were higher in all samples that were mainly by the climatic differences and alternating bearing in olive production. The mentioned microbial groups were found in the highest numbers in Hurma olives as a result of lower level of total phenolic contents in both seasons. Besides, during maturation period in both seasons, *Pseudomonas sp., Lactabacillus sp.* and *Staphylococcus sp* remained under detectable limits, so no enumeration was performed in related agar media with these microbial groups.

Physiological and biochemical characteristics were determined by phenotypic and molecular methods. The phenotypic characteristics were determined in terms of cell morphologies, cell structures by Gram-staining, catalase activity, carbohydrate fermentation, indole test, Voges-Proskauer test, nitrate reduction, motility, citrate utilization, H_2S production test. Isolates were divided into subgroups by phenotypic methods and representative cultures were picked to further molecular methods. The molecular characterization was performed by 16S DNA sequencing methods. According to DNA sequencing, identified bacterial microflora of samples were in accordance with the literature. *Bacillus sp., Pantoea sp., Acinetobacter sp., and Pseudomonas sp.,* were found in Hurma olive microflora. Non-debittered Erkence olive microflora comprised of *Bacillus sp., Pantoea sp., Klebsiella sp. and Micrococcus sp.;* leaf microflora was more diversified than the others that comprised of *Bacillus sp.,Pantoea sp., Streptomyces sp., Rhodococcus sp., Arthrobacter sp., Micrococcus sp., Curtobacterium sp.* and *Staphylococcus sp.*. The air microflora of Erkence variety olive orchards was similar to

leaf's in which, *Arthrobacter sp.*, *Bacillus sp.* (with more diversified species than the others) and *Micrococcus sp.* were presented.

It is worth noting that almost all identified bacteria from whole samples are soilborne. Because, transmission of these bacteria are occurred by air. For the further research, soil samples of orchards should be investigated in terms of determination of similarities between Hurma olive and its microflora.

As a future study, in order to compare Hurma olive's microflora, same type of olives grown in another orchards should be investigated in the same area of Karaburun to exhibit the differences or similarities between them. Moreover, biennial bearing in olive yield is common phenomena, duration of olive growth monitoring should be extended more than two years. At least four consecutive seasons (2 on seasons-2 off seasons) should be investigated with respect to enumeration of microorganism groups and determination of microflora of Hurma olive and its environmental microflora should be performed.

For commercialization of Hurma olive, packaging methods should be developed with modified athmosphere tecniques. Especially, the growth ability of molds and yeasts at low water activity renders unprocessed Hurma olive fruits susceptible to spoilage due to high initial load of mold and yeast found in Hurma olive. Besides Hurma olives would be served to the market as a healty food alternative with salt-free or non brined olives, improvement of this kind of packaging techniques is essential.

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APPENDIX A

MEDIA

A.1. Plate Count Agar (Difco 247940)

23.5 g of PCA was dissolved in 1L of distilled water and mix throughly. Heated to boil for completely dissolve abd autoclaved 121^{o} C for 15 minutes.

A.2 Potato Dextrose Agar (Difco 213400)

39 g of PDA was added into 1L of distilled water and heated with the frequent agitation and boiled to dissolve before sterilised at 121^{o} C for 15 min. After cooling of medium to 45-50 ^{o} C, to change pH 3.5, 10 mL of 10 % of sterile tartaric acid is added to medium aseptically and mixed well.

A.3 Oatmeal Agar (Difco 255210)

72.5 g of OA was added into 1L of distilled water and heated with the frequent agitation and boiled to dissolve before sterilised at 121^{o} C for 15 min.

A.4. Sabouraud Dextrose Agar (Difco 210950)

65 g of SDA was added into 1L of distilled water and heated with the frequent agitation and boiled to dissolve before sterilised at 121^{o} C for 15 min.

A.5. Czapex Dox Agar (Difco 233810)

35 g of Czapex-Dox broth and 15 g agar were added into 1L of distilled water and heated with the frequent agitation and boiled to dissolve before sterilised at 121^{o} C for 15 min.

A.6. Lactobacilli MRS Agar (Difco 288210)

70 g of MRS agar was added into 1L of distilled water and heated with the frequent agitation and boiled to dissolve before sterilised at 121^{o} C for 15 min. After sterilization, pH was adjusted to 3.5 with membrane filtered 10 % of tartaric acid solution.

A.7. Pseudomonas Agar (Oxoid CM0559)

24.2 g of Pseudomonas agar base was added in 500 ml of distilled water and bring to the boil to dissolve completely; then 5 ml of glycerol was added prior to sterilised at 121^{o} C for 15 min. When cooled to 50^{o} C, the contents of 1 vial of Pseudomonas C-N supplement (Oxoid SR102) was added and mixed well.

A.8. Nutrient Broth (Difco 234000)

8 g of nutrient broth was added into 1L of distilled water and heated with the frequent agitation and boiled to dissolve and 10 ml of dissolved nutrient broth was distributed into tubes prior to sterilised at 121^{o} C for 15 min.

A.9. Violet Red Bile Glucose Agar (Difco 218661)

41.5 g of VRBGA was added into 1L of distilled water and heated with the frequent agitation and boiled to dissolve before sterilised at 121^{o} C for 15 min.

A.10. Baird-Parker Agar (Difco 276840)

63 g Baird-Parker agar base was added into 950 ml of distilled water and heated with the frequent agitation and boiled to dissolve before sterilised at 121^{o} C for 15 min. When cooled 50 ^{o} C, 50 ml egg yolk tellurite emulsion was added and mixed well.

A.11 Bromcresol Purple Carbohydrate Broth

13 g of nutrient broth was dissolved into distilled water. After adjusting the pH to 7.2, 5 ml bromcresol purple solution were added as an indicator and the volume is completed to 900 ml with distilled water. It was followed by sterilization at 121°C for 15 min. After cooling, carbohydrate solution that has been sterilised by filter was added to the medium aseptically.

A.12 Triple Sugar Iron Agar (Merck 1.03915)

35 g of TSI agar were suspended in 1L distilled water by heating with frequently agitation and autoclaved at 121° C for 15 min.

A.13. Tryptone Water (Merck 1.10859)

15 g of tryptone water were dissolved in 1L of distilled water. 10 ml od tryptone water dispersed into tubes prior to autoclaved at 121° C for 15 min.

A.14. Motility Test Medium (Difco 211436)

22 g of motility test medium powder was suspended into 1L deionized water by heting to boil and 5 ml dispersed into tubes, autoclaved at 121° C for 15 min.

A.15. SIMMONS Citrate Agar (Merck 1.02501)

22.5 g of agar media was suspended into 1L deionized water by heting to boil and 5 ml dispersed into tubes, autoclaved at 121° C for 15 min.

A.16. Nitrate Broth (Difco 226810)

9 g of nitrate broth powder was dissolved into 1 L deionized water by heating to boil and dispersed into tubes; autoclaved at 121° C for 15 min.

A.17. MR-VP Broth (Merck 1.05712)

Suspend and mix 17 g in 1L distilled water and disperse 5 ml in tubes and autıclave 121° C for 15 min.

A.18. MacCONKEY Agar (Merck 1.05465)

50 g agar media was dissolved in 1L of distilled water by heating and autoclaved 121° C for 15 min.

A.19. Urea Broth

0.1 g of yeast extract, 9.1 g of KH_2PO_4, 9.5 of Na_2HPO_4, 20 g of urea and 0.01 g phenol red were mixed and dissolved in 1L distilled water by heating. Filter sterilized urea broth were dispersed into tubes.

APPENDIX B

CHEMICALS AND REAGENT

Table B.1 Chemicals and Reagents Used in the Experiments

Chemicals	**Brands and Codes**
Agar	Difco
D (+)Glucose anhydrous	Riedel-de Haen 16325
L (+)-Arabinose	Merck 1.01492
Sucrose	Ambresco 0335
D-Fructose	Ambresco 0226
D-Mannitol	ABCR AB114537
D-Maltose	Merck Art5911
D-Cellobiose	Sigma C-7252
Lactose monohydrate	Merck 1.07657
L(+) - Tartaric acid	Merck 1.00804
Ringers' tablet	Merck 1.15525
Glycerol	Merck 1.04092
Potassium phosphate	Applichem A2945
Immersion oil	Merck 1,04699
EDTA	AppliChem A2937
Trizma Base	Sigma T6066
2-propanol	AppliChem A3928
Bromcresol purple, indicator grade	Sigma-Aldrich 11,437-5
Ethidium bromide	Applichem A1151
Ethanol	Tekkim TK200650
Taq polymerase (recombinant)	Fermentas EP0402
dNTP set, 100mM solutions	Fermentas R0182
Agarose	Sigma A9539
Lysozyme, from chicken egg white	Fluka 62971

(Cont. on next page)

Table B.1 (Cont)

Proteinase K, from*Tritirachium album*	Sigma-Aldrich P2308
Chloroform, ultrapure	AppliChem A3633
Isoamyl alcohol	AppliChem A2610
Sodium dodecyl sulphate (SDS)	Sigma L4390
Sodium hydroxide	Merck 1.06498
Hydrochloric acid (HCl)	Sigma-Aldrich 7102
Iodine	Merck
100 bp DNA ladder,Gene Ruler TM	Fermentas SM0313
Urea	Applichem A1049
Sodium chloride	Ambresco 190
Na_2HPO_4	Riedel-de Haen 04270
KH_2PO_4	Riedel-de Haen 04243
Sephadex G-75	Sigma-Aldrich G75120
RNAs-A (DNAse free, salt free)	Applichem A3832
Cethyltremethylammoniumbromide (CTAB)	Ambresco 0833
Glacial acetic acid	Merck 1.00056
Zinc powder	Merck 1.08789
Barritts'reagent A (VP1 reagent)	Fluka 29333
Barritts'reagent B (VP2 reagent)	Fluka 39442
Kovacs' indole reagent	Merck 1.09293
Nitrate A	Remel R21536
Nitrate B	Remel R21538
Phenol red, indicator grade	Merck 1.07241

APPENDIX C

SOLUTIONS

C.1. Phosphate-Buffered Solution (1X)

8g NaCI, 0.2 g KCI, 14.4 g Na_2HPO_4, 0.24 g KH_2PO_4 were dissolved in 700 ml distilled water by frequently agitation. pH was adjusted to 7.4 using HCl. Final volume was adjusted to 1L with addition of distilled water and sterilized by autoclaving at 121° C for 20 min.

C.2. Bromcresol Purple Solution

2 g of bromcresol purple indicator was dissolved into 100 ml distilled water and solution is mixed thoroughly.

C.3. Carbohydrate Stock Solution

10g of carbohydrate was dissolved into 100 ml distilled water by stirring and filter sterilization was applied.

APPENDIX D

BUFFERS AND STOCK SOLUTIONS

D.1 1M Tris-HCl pH 7.2 and pH 8.0

121.1 g of Tris base was dissolved in 800 ml of deionized H2O. pH was adjusted to the desired value by adding concentrated HCl. The approximate values of the amount of HCl required for the desired pH values are given below.

pH	*HCl*
7.4	70 ml
7.6	60 ml
8.0	42 ml

The solution was allowed to cool to room temperature before making final adjustments to the pH, and the volume of the solution was adjusted to 1 L with H2O. The pH of Tris solutions is temperature-dependent and decreases approx. 0.03 pH units for each 1°C increase in temperature. It was dispensed into aliquots and sterilized by autoclaving. If the 1 M solution had a yellow color, it was discarded and obtained Tris of better quality.

D.2 0.5M EDTA pH 8.0

186.1 g of disodium EDTA•2H2O was added to 800 ml of deionized H2O. It was stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with 10N of NaOH (or approx. 20 g of NaOH pellets). Volume was adjusted to 1 L with deionized water. It was dispensed into aliquots and sterilized by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approx. 8.0 by the addition of NaOH.

D.3 50X TAE

242 g of Tris base was dissolved in deionized H2O. 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) were added to the solution. Lastly volume was adjusted to1 L with deionized water.

D.4 1X TAE

20ml of 50 X TAE buffer was taken and the volume was adjusted to 1 L with deionized water. The 1x working solution was 40 mM Tris-acetate/1 mM EDTA.

D.6 5M NaCl

292.2g NaCl was dissolved in deionized water and the volume was adjusted to 1L.

D.7 Ethidium Bromide Stock Solution (10mg/ml)

0.5g ethidium bromide was dissolved in 50ml deionized water and the solution was storred in dark bottle at room temperature.

D.8 Chloroform-Isoamyl Alcohol Solution

In proportion of 24:1, chloroform:isoamylalcohol were mixed.

D.10 1 X TE BUFFER

100mM Tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0) was mixed and the buffer was stored at room temperature.

D.11 CTAB/NaCl Solution

4.1g NaCl was dissolved in 80ml deionized water. 10g CTAB was added slowly while heating and stirring. The solution can be heated to 65°C to increase the dissolution. Lastly, the final volume was adjusted to 100ml.

D.12 10% Sodium Dodecyl Sulfate (SDS)

100g of SDS was dissolved in 900ml of deionized water. Solution was heated to 68°C to dissolve. The pH was adjusted to 7.2 with the addition of a few drops of concentrated HCl. The volume was adjusted to 1L with deionized water.